The glutathione/glutathione-related enzyme system in reproduction

Maarten Knapen

THE GLUTATHIONE / GLUTATHIONE-RELATED ENZYME SYSTEM IN REPRODUCTION

Maarten F.C.M. Knapen

THE GLUTATHIONE / GLUTATHIONE-RELATED ENZYME SYSTEM IN REPRODUCTION

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CONTENTS.

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CI	Confidence Interval
EC	Enzyme Commission
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
17β-E_2	17β-Oestradiol
FSH	Follicle Stimulating Hormone
GPX	Glutathione Peroxidase
SeGPX	Selenium dependent Glutathione Peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
GSTA	Glutathione S-transferase Alpha
GSTA1-1	Glutathione S-transferase Alpha 1-1
GSTM	Glutathione S-transferase Mu
GSTP	Glutathione S-transferase Pi
GSTP1-1	Glutathione S-transferase Pi 1-1
GSTT	Glutathione S-transferase Theta
hCG	Human Chorionic Gonadotropin
HPLC	High Performance Liquid Chromatography
HRT	Hormone Replacement Therapy
IVF	In Vitro Fertilisation
LDH	Lactate dehydrogenase
LH	Luteinizing Hormone
n	Number
P	Progesterone
PBS	Phosphate-buffered saline

CHAPTER 1

Aim of the thesis

Hypertensive disorders are serious complications of pregnancy, with high perinatal morbidity and mortality. The etiology of these diseases is still largely unknown Circulating 'toxic substances', lipid peroxides or oxygen free radicals generated by the feto-maternal interface may lead to maternal endothelial damage and dysfunction The resulting maternal clinical syndrome may include a variety of pathophysiological features such as vasocontriction, decreased plasma volume, haemolysis and multi-organ failure Recent studies have focussed their attention on a possible deficient maternal detoxicating system, unable to deal with the increased load of 'toxic substances'.

The glutathione / glutathione-related enzyme system in a quantitative sense is one of the most important detoxicating systems in humans Glutathione and glutathione-related enzymes (glutathione S-transferases and glutathione peroxidases) are involved in the detoxification of endogenous and exogenous toxic substances, carcinogens, and oxygen radicals. Deficiencies in parts of this system may be related to the etiology of hypertensive disorders of pregnancy. Serum or plasma concentrations of glutathione S-transferases may be reliable markers for disease. For instance, plasma glutathione S-transferase Alpha 1-1 is a sensitive and specific marker for hepatocellular impairment, and glutathione S-transferase Pi 1-1 may be a marker for haemolysis.

In this thesis several studies are presented on the glutathione / glutathione-related enzyme system in reproduction, mainly focusing on hypertensive disorders of pregnancy

CHAPTER 2

Glutathione and glutathione-related enzymes in reproduction a review

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ABSTRACT.

The glutathione / glutathione-related enzyme system in a quantitative sense, is one of the most important protective systems in humans. Glutathione and glutathione-related enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds as well as reactive oxygen species. The role of reactive oxygen species in reproduction has been the subject of many investigations and there is compelling evidence for the involvement of these species in the physiology and pathology of both male and female reproductive systems. The glutathione / glutathione-related enzyme system is extensively studied in gynaecological oncology, but to a lesser extent in other fields related to reproduction. In this paper a review is provided on the glutathione / glutathione-related enzyme system in reproduction. Attention is given to its role as a detoxicating system and as an early marker for disease.

1. INTRODUCTION.

The glutathione / glutathione-related enzyme system (GSH / GSH-related enzyme system), in a quantitative sense, is one of the most important protective systems in humans. GSH and GSH-related enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds as well as reactive oxygen species (ROS) [1]. The role of ROS in reproduction has been the subject of many investigations, and there is compelling evidence for the involvement of ROS in the physiology and pathology of both male and female reproductive systems [2, 3]. The GSH-related enzymes, glutathione peroxidase (GPX) [4] and glutathione S-transferase (GST) are also involved in the metabolism of leukotrienes and prostaglandins [5]. Serum or plasma GST levels are measured as markers of disease [6]. The GSH / GSH-related enzyme system has been studied extensively in oncological disease [7] [for reviews see: Hayes [8, 9], Beckett [6], Tsuchida [10]] So far relatively little attention has been given to the study of this important detoxicating and scavenging system in the etiology and pathophysiology of reproduction-related non-oncological disease, such as hypertensive disorders of pregnancy [11]. Until now this research has been focused mainly on GPX in relation to scavenging and lipid peroxide inactivating functions [11]

In this chapter a review is provided of the GSH / GSH-related enzyme system in reproduction. Attention is given to its role as a detoxicating system, and as an early

marker for disease.

2. DETOXIFICATION MECHANISMS.

Reactive oxygen species (ROS) or oxygen radicals are involved in human disease [12, 13]. Most, if not all, organisms are equipped with enzymatic and non-enzymatic defence mechanisms against oxidants and other toxic compounds Many toxic compounds are ingested through nutrition, air pollution *etc* , or formed within the body [14]. Enzymatic protection against ROS and the breakdown products of peroxidized lipids and oxidized DNA is provided by superoxide dismutases [15], catalase [15], GPX [16], GST [17], aldo ketoreductases [18] and DNA repair enzymes [19] Non-enzymatic detoxification is provided by many different agents such as transferrin, ceruloplasmin, lactoferrin, vitamin E (α-tocopherol), vitamin C (ascorbic acid), uric acid and taurine, as well as thiols, such as GSH, cysteamine and cysteine. The GSH / GSH-related enzyme system, in a quantitative sense, is one of the most important protective systems in humans.

2.1. GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES.

2.1.1. Glutathione.

The tripeptide glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine, *Fig. 1*) plays a central role in the protection of cells against oxidative [20, 21] and electrophilic [22, 23, 24] stress and radiation [25] *Oxidative stress* refers to the unusually high presence of molecules with a high potency to abstract electrons from biomolecules Free radicals are the most important class of biological oxidators *Electrophilic stress* is defined as the effect of compounds with a high reactivity towards nucleophilic centres present in biomolecules They are with increasing reactivity oxygen, nitrogen and sulphur This leads to the formation of new chemical bonds between the two (adduct formation) GSH can act either as a substrate in the cytosolic GSH-redox cycle, or is able to directly inactivate ROS, such as O_2 and OH Erythrocytes and hepatocellular tissue are thought to contain the highest GSH redox activity [26] GSH is the most abundant non protein sulfhydryl present in cells, with cellular concentrations ranging from 0 5 - 10 mM, most of which exists in the reduced form [27]. The synthesis of GSH by aerobic cells can be considered as a way to hold high levels of usable cysteine in a non-toxic form Cysteine itself will rapidly auto-oxidize in the presence of the metal ions Fe^{3+} and Cu^{2+} [28]

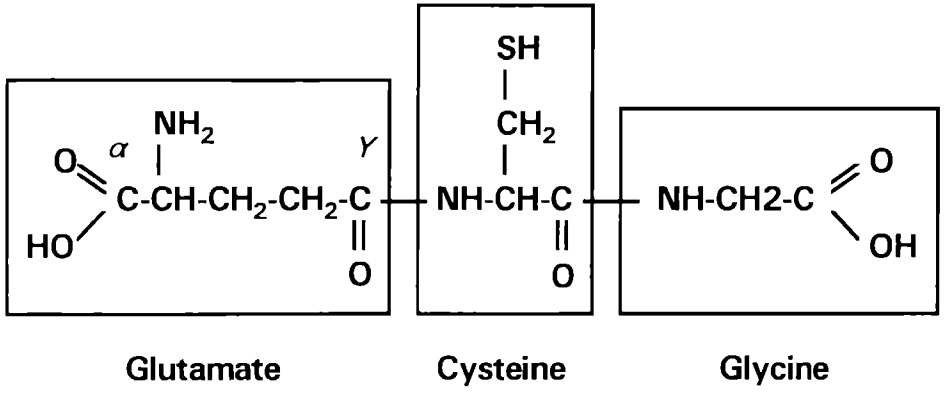

Fig 1 *The structure of the tripeptide glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine)*

As a result of the hydrogen peroxides liberated during this auto-oxidation simple thiols such as cysteamine [29, 30] and cysteine [30] can be toxic to cells Blocking of the cysteine amino group by forming γ-glutamyl-cysteine considerably slows this auto-oxidation [31] Coupling of glycine to the γ-glutamylcysteine dipeptide by GSH synthetase leading to GSH synthesis, makes it even more resistant to auto-oxidation [31] Furthermore, this reaction provides a more extended carboxylsite that is used for substrate recognition and active site handling by many GSH dependent enzymes [32, 33] The abundance of GSH in biological systems has led to an enormous amount of research to find 'the role of GSH' [32] Currently a myriad of functions is known, and many of them are vital Some of these important functions are (a) the detoxification of xenobiotics [34], (b) removal of hydrogen peroxide and other peroxides as well as free radicals [21], (c) maintenance of free protein sulfhydryl groups [35] and (d) the synthesis of leukotriene C_4 and derivatives [36]. These functions may lead to variations in the cellular availability of GSH [37] Under conditions of marked toxicity or oxidative stress, a variety of processes can occur which may lead to the oxidation of GSH into the oxidized form GSSG [38, 39] Regeneration of GSH from oxidized GSH in the cell is affected by the enzyme GSH reductase Due to the high nucleophilic potency of the GSH sulfhydryl moiety, GSH can function as an excellent nucleophilic scavenger for many reactive electrophiles [22, 33] In many cases this reactivity is further increased by GST [34] As electrophilic chemicals can also react with biological macromolecules

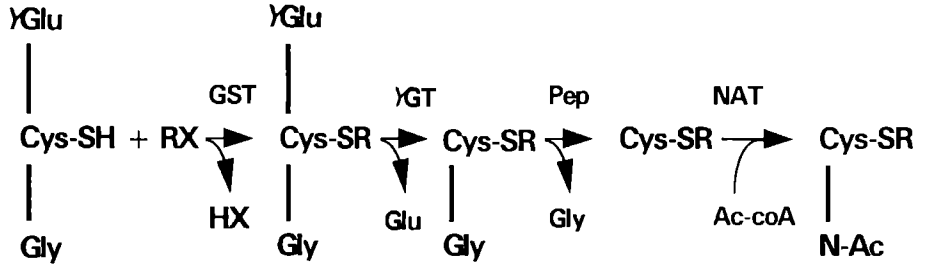

Fig 2 *Glutathione conjugation by glutathione S-transferase (GST) and subsequent steps of the mercapturic acid pathway The γ-glutamyl moiety is removed by γ glutamyl transferase (γGT) the remaining dipeptide can subsequently be cleaved by cysteinyl glycine dipeptidase or aminopeptidase M to yield a cysteine conjugate This cysteine conjugate can be N-acetylated by N-acetyl transferase (NAT), which leads to mercapturic acid formation*

such as DNA, thereby presenting a genotoxic risk [40], this conjugation with GSH furnishes an important protection mechanism In some instances however, the GSH conjugates may in turn be toxic to cells [41, 42, 43] GSH S-conjugates can be further metabolized to the corresponding cysteine conjugates by γGT, cysteinylglycine dipeptidase and aminopeptidase M [42], which can subsequently be N-acetylated to yield mercapturic acids (*Fig 2*) [44, 45] The thio-ethers formed are often excreted in urine and can be used for biological monitoring of exposure to electrophilic compounds [45, 46]. Formation of GSH conjugates will lead to loss of available GSH. For an overall summary of GSH metabolism see *Fig. 3.*

2.1.2. Glutathione S-transferases.

The metabolism of foreign compounds usually involves two distinct stages, commonly referred to as phases I and II Phase I metabolism involves an initial oxidation, reduction or dealkylation of the xenobiotic by cytochrome P450 (CYP) monooxygenases [47] This step is often needed to provide a molecule with hydroxyl- or amino-groups, which are essential for phase II reactions. Phase II metabolism generally adds hydrophilic moieties, thereby making a toxin more water soluble and less biologically active Most involved phase II conjugation reactions are catalyzed by GST [34], UDP glucuronyl transferases [48], and sulfotransferases [49] The GSTs catalyze the addition of GSH to a wide variety of exogenous compounds such as carcinogens, toxins and drugs,

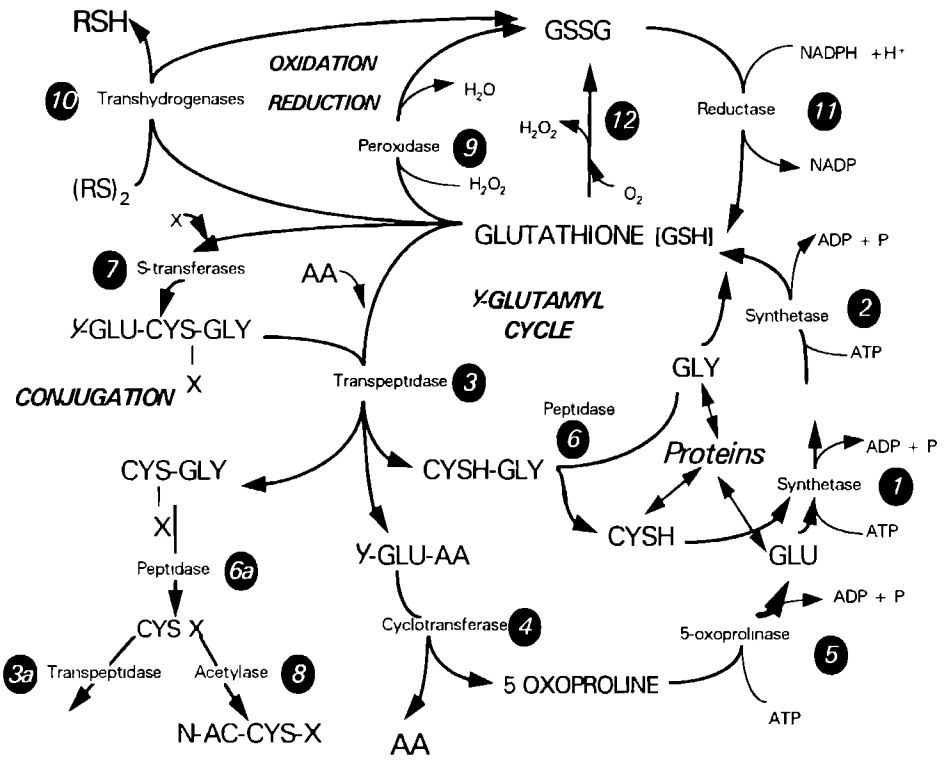

Fig 3 *Overall summary of glutathione metabolism reaction 1 γ-GLU CYSH synthetase Reaction 2 GSH synthetase, Reaction 3 and 3a glutamyl transpeptidase, Reaction 4 γ-glutamyl cyclotransferase, Reaction 5 5-oxoprolinase, Reaction 6 and 6a dipeptidase, Reaction 7 GSH transferases, Reaction 8 N-acetylase, Reaction 9 GSH peroxidase, Reaction 10 transhydrogenases, Reaction 11 GSSG reductase, Reaction 12 oxidation of GSH by O_2 Conversion of GSH to GSSG is also mediated by oxygen free radicals*

resulting in the formation of mercapturic acids [1]

The cytosolic glutathione S-transferases (EC 2 5.1 18) are a family of enzymes consisting of four main classes, Alpha, Mu, Pi and Theta, each divided into one or more isoforms [1, 6] They provide protection against electrophiles and products of oxidative stress and can also serve as isomerases [34] The insensitivity of cells to chemical stress is dependent on the levels of expression of the chemical defence mechanisms mentioned in the first lines of this paragraph Protection against oxidative stress is partly provided by the peroxidase function of certain GST isoenzymes by catalyzing the

reduction of organic hydroperoxides to their corresponding alcohols [50] Several GST isoenzymes possess selenium-independent GSH peroxidase activity with organic hydroperoxides such as linoleic and arachidonic hydroperoxide, but not with hydrogen peroxide [51] The class Alpha GSTs exhibit the highest peroxidase activities [9] It should be noted that endogenous compounds such as leukotriene C_4 [52] and prostaglandin H_2 [5, 53] are also metabolized by GSTs as part of their normal biosynthetic pathways All GSTs possess the ability to sequester substrate or non-substrate drugs, hormones [54], including steroid and thyroid hormones, bile acids, bilirubin, fatty acids, haemoglobin, penicillin, and many other compounds [9], serving as main intracellular transport proteins

GSTs are widely distributed in nature and all eukaryotes appear to possess multiple isoenzymes [9] Many molecular forms of GSTs have been identified from various organs in a variety of species [6, 9, 34, 55, 56] Humans possess multiple cytosolic GST-isoenzymes, each of which displays distinct catalytic as well as non-catalytic binding properties The cytosolic enzymes are encoded by at least four related gene families (designated class Alpha, Mu, Pi and Theta GST [57, 58, 59]), whereas the membrane-bound enzymes, microsomal GST and leukotriene C_4 synthetase are encoded by single genes and both have arisen separately from the soluble GSTs Generally, GSTs that share more than 40% identity belong to the same class and those that possess less than 30% identity are assigned to separate classes In humans, class Alpha, Mu, Pi and Theta GST genes are located on chromosomes 6, 1, 11 and 22 respectively [9] The cytosolic GSTs are dimeric proteins, homodimers or heterodimers consisting of two subunits of approximately 25 kD molecular mass (range 23 - 28 kD) [60] The distribution of GST isoenzymes is tissue specific The reasons for this specific expression of GST isoenzymes most likely reflects the need to deal with specific substrates, which considerably vary between organs GST class Alpha is mainly expressed in the liver but is also present in the kidney, testis, adrenal gland and small intestine [1, 7, 60, 61, 62, 63] Two class Alpha subunits have been identified and two homodimeric (GSTA1-1 and GSTA2 2) and a heterodimeric enzyme (GSTA1-2) have been purified from human liver [64] For GST Pi, so far one isoform is described GSTP1-1, which is expressed as a major form in many organs such as lung, breast, placenta, erythrocytes, large intestine or urinary bladder [7, 65, 66, 67, 68] The molecular evidence of allelopolymorphism of the human GST Pi gene locus, resulting in active, functionally different GST Pi proteins was reported recently [69] The human Mu and Theta classes of GST appear to be expressed at relatively low levels in many organs, Mu has been found in the liver, while other subtypes that are not expressed in

the liver are found in muscle, liver, testis and brain [9]. A GSTM1-1 null genotype was found to be present in approximately 50% of Caucasians tested [67, 70, 71]. Two class Theta GSTs have been purified: GSTT1-1 and GSTT2-2, of which class Theta1-1 is polymorphic [72]; a GSTT1-1 null genotype was shown to be present in 15% of Caucasians [73]. Both percentages greatly vary between the different human races [6, 73]. Other polymorphisms in the Mu and Theta genes have been reported [74, 75] The biological consequences of failure to express GSTM1-1 or GSTT1-1 enzymes can include increased susceptibility to bladder [76], colon [77], skin [78] or lung cancer [79].

The biological control of the cytosolic families is complex as they exhibit sex, age-, tissue-, species- and tumour-specific patterns of expression [9]. In addition, GSTs are regulated by a structurally diverse range of xenobiotics of which a significant number is consumed as naturally occurring non-nutrient components of vegetables and citrus fruits Many of the compounds that induce GST themselves are substrates for these enzymes or are metabolized to compounds that can serve as GST substrates, suggesting that induction of GST represents part of an adaptive response mechanism to chemical stress caused by electrophiles in the diet. In addition, ROS such as superoxide radical, hydrogen peroxide, and the hydroxyl radical may arise as a consequence of aerobic respiration, ionizing irradiation, inflammation or cellular response towards invaders (i e respiratory burst) These potentially harmful compounds may interact with membrane lipids and DNA [80].

A high expression of GSTP1-1 has been found in many human tumours, including carcinomas of the colon, lung, kidney, ovary, pancreas, oesophagus, and stomach [7, 68, 81, 82] Several human tumour cell lines over express GSTP1-1, although incidently high levels of class Alpha and Mu isoenzymes are also observed [10, 83]. On the other hand, in ovarian tumours, amongst others, low GSTP1-1 levels have been reported, associated with responsiveness to chemotherapy [84, 85]. Measurements of plasma GSTP1-1 levels have shown that this protein may be dramatically increased in cancer patients, but the extent of over expression of this protein in many tumours is frequently insufficient to allow the enzyme to serve as a tumour marker [86] Additionally, methodological problems may give falsely high serum or plasma GSTP1-1 levels [86]. Measurement of GSTP1-1 has been advocated as a marker for haemolysis, because of its abundant presence in erythrocytes [87]. Plasma GST Alpha has been postulated as a superior marker for hepatocellular impairment. In contrast with the aminotransferases (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]), which are found predominantly in the periportal hepatocytes, GST Alpha is distributed uniformly

across the liver lobule and is present in high amounts [88]. The enzyme is rapidly released into the bloodstream during hepatocellular impairment [6]. In patients with paracetamol poisoning, halothane hepatitis or rejection following liver transplantation, plasma GST Alpha concentrations are known to provide a more sensitive index of acute hepatocellular impairment than AST or ALT activity [6, 89, 90, 91, 92]. Furthermore, when the active phase of hepatocellular impairment is over, plasma concentrations of GST Alpha revert more rapidly to normal, a feature of the short half-life (under 90 minutes [92]), whereas aminotransferase activity may be elevated much longer.

2.1.3. Glutathione peroxidases.

Glutathione peroxidases (GPX, EC 1.11.1.9) are enzymes that catalyze the reduction of organic hydroperoxides (lipid hydroperoxides, DNA hydroperoxides) or hydrogen peroxide [93] by GSH. GPX has been demonstrated in all mammalian tissues examined [94]. Two major types of GPX have been found. One type is distinguished by containing selenium in the form of a covalently bound selenocysteine in its active site (SeGPX) and is active with both organic hydroperoxides and hydrogen peroxide [95, 96]. The second type of GPX consists of proteins that do not depend on selenium for catalysis and have negligible activity with hydrogen peroxide. This type comprises mainly of GST [97]. The peroxidase activity of the GSTs may represent a major biological function in protection against oxidative stress [98]. In selenium deficient states, the relative importance of the peroxidase activity of the GSTs may even increase [97].

Selenium-dependent GPX activity is due to the expression of multiple isozymes. At least four of the isozymes has been purified and characterized: (a) the classical cellular GPX, GPX-1 [95], (b) the phospholipid hydroperoxide GPX, PHGPX [99], (c) the plasma GPX, GPX-P [100, 101]. Both GPX-1 and GPX-P are tetramers and have similar substrate specificities. They are able to metabolize hydrogen peroxide (H_2O_2) and fatty acid hydroperoxides effectively, but metabolization of the hydroperoxides of phospholipids and cholesterol is poor [102, 103]. PHGPX is a monomer and has different substrate specificities as compared to GPX-1 and GPX-P. PHGPX efficiently catalyzes the reduction of hydroperoxide of phospholipids or cholesterol and linoleic acid much more effectively than the reduction of hydrogen peroxide or *tert*-butyl hydroperoxide. A fourth (d) selenium-dependent GPX, GPX-GI has recently been demonstrated. GPX-GI has similar substrate specificities as GPX-1. They both catalyze the reduction of hydrogen peroxide, *tert*-butyl hydroperoxide, cumene hydroperoxide,

and linoleic acid hydroperoxide, but not of phosphatidylcholine hydroperoxide. GPX-GI is mainly located in gastrointestinal tissue (liver and colon) and occasionally in human breast tissue [104].

3. GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES AND FOLLICULAR MICRO-ENVIRONMENT.

Only a few studies have been published concerning the GSH / GSH-related enzyme system in the ovarian follicle and the follicular micro environment. Because inhibition of ROS leads to inhibition of ovulation, it is thought that ROS are released in connection with follicle rupture [105]. ROS are also thought to play a role in oocyte maturation [2]. In a recent study, GSTP1-1 and GSTA1-1 were demonstrated in considerable amounts in the human ovarian follicular fluid of patients participating in an *in vitro* fertilization (IVF) programme [106, *Chapter 3*]. Notorious xenobiotics such as polychlorinated biphenyls (PCBs) [107, 108] and hexachlorobenzene (HCB) [109] were demonstrated in the follicular fluid of IVF patients These xenobiotics are known to induce GST in tissues other than ovaries [110, 111]. The presence of GSTA1-1 and GSTP1-1 in ovarian follicular fluid may suggest a role as detoxifying enzymes to protect the ovum from ROS and xenobiotics [106, *Chapter 3*]. Unfortunately, no data on GSH levels in follicles or its micro-environment are available so far.

The presence of selenium and SeGPX has been demonstrated in follicular fluid [112]. Levels of selenium or SeGPX have been related to disorders of fertility Patients with unexplained infertility showed significantly lower follicular selenium levels as compared to those with tubal infertility or infertility due to male factors. The mean SeGPX activity in follicles yielding oocytes that were subsequently fertilized, were higher than those of follicles with non-fertilized oocytes. Interestingly, tobacco smoking has been shown to be associated with significantly diminished follicular SeGPX activity [112]. These results suggest that SeGPX acts as a protective enzyme for oocytes.

In summary, detectable amounts of SeGPX, GSTA1-1 and GSTP1-1 have been demonstrated in human ovarian follicular fluid. The most important finding is that SeGPX activity is higher in follicles yielding oocytes that are subsequently fertilized as compared to follicles with subsequently non-fertilized oocytes.

4. GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES IN ASSISTED REPRODUCTION.

Numerous studies, mainly animal experiments, have described the role of GSH and

GSH-related enzymes in assisted reproduction Attention has been focused on male reproduction, reproductive performance and the influence of the exposure of carcinogens on semen parameters Additionally, several studies have highlighted the role of GSH and GSH-related enzymes in relation to *in vitro* fertilization and *in vitro* embryo development.

4.1. Male reproductive physiology and pathology.

Glutathione depletion of several male reproductive tissues can be achieved by administration of specific glutathione depleting agents and this may affect reproductive outcome. Depletion of GSH in male rat reproductive tissues, such as cauda and caput epididymis by means of 1,2-dibromoethane injection is correlated with an increase of dominant lethal mutations in their offspring [113] Fluoride administration to rats has resulted in significantly lower GSH levels in testis and especially cauda epididymis [114] Intratesticular injection of GSH depleting agents (buthionine sulfoximide (BSO) or diethylmaleate) has resulted in selectively lowered testicular GSH levels However, no changes have been observed in testicular spermatid count or in the morphology or motility of cauda epididymal sperm [115] GSH depletion potentiates ethyl methanesulfonate-induced alterations of sperm chromatin structures, that are likely to be related to dominant lethal mutations [116] In the reproductive tissue of bulls, testis, caput epididymis and ampulla have shown the highest levels of GSH, whereas the GSH content of washed caput epididymal sperm is three times the GSH content of cauda epididymal or ejaculated sperm [117]. In spermatozoa, GSH has shown maximal staining in the midpiece and tail regions [117]. The highest level of γ-glutamyltranspeptidase (γGT) has been encountered in the epididymis [117]. Administration of the GSH precursor N-acetylcysteine to male rats, prior to challenge with ethyl-methanesulfonate has resulted in a significant decrease in post implantation loss, indicating a restoration of reproductive performance [118]

GSTs are differentially expressed in testicular compartments and epididymal segments of rats, possibly resulting in a characteristic susceptibility of different celltypes to xenobiotic damage [119] GSTs have also been identified in different types of human testicular cells They may play a role in normal human spermatogenesis and may protect germ cells from teratogens and carcinogens [120] GSTA1-1 and GSTP1-1 have been demonstrated in human seminal fluid [Knapen, unpublished results].

GSH reductase [121, 122], GPX [121, 122], and GSH [121] have been found in human spermatozoa, but they show little variation amongst samples GPX and GSH

reductase have also been identified in human seminal plasma [123] In the seminal plasma of bulls, it has been shown that GPX activity decreases with increasing paternal age [124] Sperm GPX and GSH reductase activities remain unchanged after cryopreservation, though enhanced levels of lipid peroxidation products have been found [125]. After liquid storage of sperm, an improvement of survival has been reported by adding increasing doses of GPX [126] From studies in mice it has been suggested that GPX in male reproductive tissue may play an important role in sperm maturation from the the very start up to the end of maturation [127] Male rat reproductive tissue expresses at least three different types of GPX [128]. GSH and GSH-related enzymes may play a role in sperm quality. Antioxidants such as GSH and N-acetylcysteine can protect against the damaging effect of leucocyte-derived ROS on sperm movement and may be of clinical value in assisted conception procedures [129]. Systemic supplementation of reduced GSH to patients with dyspermia due to varicocele or germ-free genital tract infection results in improved sperm parameters and its cell membrane characteristics [130] GPX added to semen samples of rams has positive effects on sperm motility and acrosome integrity [126] Selenium and vitamin E supplementation improves sperm motility and the percentage of normal spermatozoa in infertile men [131]

In summary, GSH and GSH-related enzymes play a significant role in the protection of (developing) sperm from carcinogens, and administration of N-acetylcysteine, GSH and GPX seems to improve sperm quality

4.2. *In vitro* fertilization and *in vitro* embryo development.

Recently, Kamrin *et al* [132] have reviewed the role of GSH in the protection of the conceptus against xenobiotics It has been demonstrated that GSH synthesis, stimulated by cysteamine and cysteine, occurs intracellularly during *in vitro* maturation of oocytes [133] It has been demonstrated that sperm gamma glutamyl transpeptidase (γGT) may be a limiting factor for male pronuclear formation in polyspermic oocytes [134] γGT is present on the surface of boar spermatozoa and male pronuclear formation has been inhibited in γGT injected pig oocytes, which suggests that sperm γGT may be a limiting factor for male pronuclear formation in polyspermic oocytes [134] GSH is present in the oocyte and it has been implicated in the reduction of disulfide bonds in the sperm nucleus during fertilization and thus in the development of the male pronucleus [135]

The addition of BSO, an inhibitor of GSH synthesis, to culture media of fertilized

oocytes has resulted in lower numbers of blastocysts formed [136]. In accordance with these results, it has been demonstrated that GSH administration to medium could prevent embryotoxicity by 2-nitrosofluorene (NOF) through non-enzymatic formation of GSH-NOF adducts [137]. The percentage of bovine embryos that reach the blastocyst stage is significantly higher when cysteamine is added to the culture medium [138] Addition of cysteamine also increases GSH levels in oocytes, suggesting that the beneficial effects of cysteamine on *in vitro* maturation and subsequent embryo development after *in vitro* fertilization is mediated by GSH [138] The addition of low-molecular weight thiol compounds (beta-mercaptoethanol or, again, cysteamine) to the culture medium improves the *in vitro* development of bovine embryos, again mediated through enhanced intracellular GSH levels [139]

Decreased embryonic total GSH levels have been observed in rat embryos of diabetic female rats The decrease is possibly a consequence of altered GSH transport across the yolk sac endodermal cells, which may be impaired under diabetic conditions The observed decreased embryonic total GSH levels may impair the protection against oxidative stress, as described in diabetic pathology [140]. Inhibition of γGT in rat embryos undergoing organogenesis can produce alterations in GSH levels and elicit embryotoxic effects [141] Pre-treatment with GSH provides protection against cadmium-induced embryotoxicity [142]

In summary, enhancement of GSH and GSH-related enzymes in oocytes is likely to improve fertilization rates in IVF procedures and seems to protect the developing embryo from carcinogens and oxidative stress

5. GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES AND THE RISK FOR RECURRENT EARLY PREGNANCY LOSS.

Environmental toxins are likely to be a cause for recurrent early pregnancy loss [143] A (partly) deficient decidual or systemic detoxification system, resulting in an increased exposure of the conceptus to endogenous or exogenous toxins, could theoretically lead to recurrent early pregnancy loss Therefore, deficient GSH or GSH-related enzymes may contribute to the etiology of this condition Erythrocyte GPX levels in women with recurrent early pregnancy loss have not shown any difference from healthy controls [144] Recently, Hirvonen *et al* have reported an increased incidence of the GSTM1-1 null genotype in a group of 29 women with recurrent early pregnancy loss as compared to the incidence in women with normal pregnancy outcomes [145] Unfortunately, they could not reproduce these results in another population group [145].

In summary, alterations in erythrocyte GPX activity or a deviant incidence of the null genotype of GSTM1-1 do not seem to be of significant importance to the etiology of recurrent early pregnancy loss Recently we were able to demonstrate an enhanced incidence of a polymorphic GSTP1 1 gene resulting in a reduction of GSTP1-1 enzyme activity, in a group of women with recurrent early pregnancy loss (Zusterzeel *et al*, unpublished results).

6. DEVELOPMENTAL ASPECTS OF GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES.

Although the distribution of GST isoenzymes in human newborn or adult tissues has been actively studied [6, 9, 146], little is known about embryonic and fetal tissues Studies of these tissues, however, are of particular importance, because several drugs and chemicals may cross the placenta and accumulate in the fetus [147, 148] Xenobiotics crossing the placenta may directly reach the fetal organs (through the venous duct), bypassing the liver [147] In the human fetus and neonate, active drug-metabolizing enzymes are present, except for some forms of UDP-glucuronyltransferase, which develop after birth [149] Several components of the maternal diet are involved in in-utero regulation of enzymes involved in GSH metabolism [150] A combination of genetic, developmental and environmental factors seems to control fetal enzyme activities and may ultimately determine the variability in inter-individual susceptibility to chemicals *in utero* [151]

The GSH levels in human fetal liver, brain and adrenal tissue have shown a decrease during late gestation The decline in the brain occurrs at an earlier gestational age Fetal GSH levels of parents with low incomes have been found to be lower than those in high income groups [152]. Fetal GST activities in liver, kidney, lung, muscle, heart, adrenal glands, pancreas, and stomach have been demonstrated to be higher than those of adults [153].

In fetal liver GSTs of class Alpha, Mu and Pi, but not Theta have been demonstrated [154] In both neonatal and adult hepatocellular tissue only GST Alpha and GST Mu are present, whereas GSTP1-1 is predominant in fetal hepatic tissue at 8-12 weeks' gestational age [155, 156] In both neonatal and adult biliary duct epithelium only GSTP1-1 is expressed, suggesting that during gestation the GST Pi locus is downregulated in hepatocytes but not in the epithelium of biliary ducts [155] The expression of Alpha and Mu class GST isoenzymes in liver tissue increases significantly at birth [157]. In contrast to these developmental differences in GST expression, it has been shown that liver GPX activities do not change appreciably from 10 weeks'

gestational age to 3 months after birth [158] Interestingly, malathion has been shown to cause alterations in the levels of GSH-related enzymes and antioxidant systems in *in vitro* studies of human fetal tissue, the effects being highest in the early period of development [159].

Both GST Alpha and Pi are expressed along the entire developing nephron of the fetal kidney, until about 35 weeks' gestational age [161] After 35 weeks, the expression of GST Alpha is restricted to the proximal tubule The expression of GSTP1-1 is restricted to the collecting tubules, as well as the distal part of the loop of Henle [160, 161] Strange *et al* have shown that before 20 weeks' gestational age the collecting tubes and primitive Bowman's capsules express GST Alpha, whereas primitive glomeruli and mesenchymal tissue do not [160] However, in other studies mainly GSTP1-1 staining has been found, whereas in meso- and metanephric tissue GST Alpha staining is absent [156, 162]. The expression of these enzymes in nephric tissue and especially in collecting tubules may point to a role in detoxification of urinary compounds excreted by the embryo [163] The expression of Alpha class GST enzymes in fetal kidney increases significantly after 40 weeks' gestational age [164], whilst the expression of class Mu and Pi GST isoenzymes do not change significantly throughout further postnatal development [164].

For the fetal lung it has been demonstrated that GSTP1-1 is down regulated during the first half of gestation [164, 165] and appears to precede phenotypic differentiation of the distal airways [160] Early on in gestation, GSTP1-1 is present in epithelial cells, but as development proceeds, expression is maintained in proximal but not in more distal airway cells [160] For GST Alpha a weak immunostaining is present in the first half of pregnancy [165, 160], being stable throughout development [164, 165] The expression of GST Mu isoenzymes is also continuous throughout development [164] Lung GPX activities do not change appreciably from 10 weeks' gestational age until 3 months after birth [158, 165]. The presence of GSTs in an early stage of development suggests an additional role of these enzymes other than the protection of the respiratory tract against air pollutants after birth

A comparison of the expression of the GSTs in developing lung, kidney and liver shows some common patterns of expression, suggesting that these genes are under similar regulatory control [164].

GSTP1-1 in the fetal brain is localized at the site of the blood-cerebrospinal fluid (CSF) barrier, blood-brain, CSF brain and pia-arachnoid-brain barrier [156, 166] In a recent study, GST Alpha immunoreactivity has been shown to be confined to a weak staining of epithelial cells in the tela chorioidea [156, *Chapter 4*] The GST isoenzymes

are well localized to prevent neuronal exposure to potentially toxic substances delivered from blood or CSF This indicates that the expression of GST Alpha and Pi this early in gestation may be highly significant in the protection of the developing human brain *In vitro* studies of human fetal brain have demonstrated that malathion causes alterations in the levels of GSH-related enzymes and other antioxidant systems These alterations were at their highest early in development, suggesting a higher susceptibility of tissues in this period [159]. An impaired responsiveness to oxidative stress of the embryonic GSH / GSH-related enzyme system is supposed to play a crucial role in oxygen-induced embryopathy and may even result in neural tube defects [167]. It has been demonstrated that the addition of valproic acid, cytochalasin and 7-hydroxy-2-acetylaminofluorene to rat embryo cultures causes increased incidences of neural tube defects, which can be diminished by the addition of the cysteine pro-drug 2-oxothiazolidine-4-carboxylate. Increased incidences of neural tube defects are noted after depletion of GSH by adding BSO to the culture medium [168] Amnionic γGT concentrations in pregnancies bearing fetus with neural tube defects do not differ from those in uncomplicated pregnancies [169]. Conflicting reports have been published with respect to erythrocyte GPX activities of parents having children with neural tube defects Hinks *et al* have not found a difference in maternal whole blood GPX activities during pregnancy in mothers with babies with neural tube defects as compared to mothers with normal offspring [170] Graf *et al.*, however, have found a low erythrocyte GPX activity in the majority of parents of a child with a neural tube defect [171] Children with myelomeningocele [171], and both high (thoracic) and mid (lumbar) [172] neural tube defects show lower erythrocyte GPX activity as compared to healthy control children.

To our knowledge, only one histological study on the expression of GST Alpha and GSTP1-1 in several embryonic gastrointestinal tissues, other than the liver has been performed until now [156, *Chapter 4*]. Van Lieshout *et al.*, have demonstrated the expression of both isoenzymes along the embryonic gastro-intestinal tract at eight weeks' gestational age. This expression is shown to be different from that in adults, because both GST Alpha and GST P1-1 are demonstrated in all embryonic epithelial cells [156, *Chapter 4*]. These isoenzymes are down-regulated selectively in some types of cells later in gestation or probably after birth. The expression of GST Alpha and Pi along the digestive tract of embryos, fetus and adults may stress the role of these proteins in the elimination of toxic and carcinogenic compounds. In embryonic pancreatic tissue, GSTP1-1 but not GST Alpha staining has been demonstrated [156, *Chapter 4*]

No developmental trends in the expression of Alpha, Mu and Pi isoenzymes of GST are demonstrated in fetal heart and diaphragm [173].

GPX activities reach adult levels in fetal erythrocytes between 26 to 35 weeks' gestational age, and are lower before the 26th week [174]. In another study however, both in preterm and term neonates, erythrocyte GPX activity is shown to be less than the activity in adult erythrocytes [175].

In summary, GSH and GSH-related enzymes are well developed in embryonic and fetal life. GST tissue expression is often different in adults. There may be a role for this system in the genesis of neural tube defects and in the detoxification of noxious compounds that cros the placenta or are generated intracellularly.

7. GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES IN HYPERTENSIVE DISORDERS OF PREGNANCY.

7.1 GSH and related enzymes in the etiology of hypertensive disorders of pregnancy.

Hypertension complicates 6 - 20 % of all pregnancies and ranks amongst the four most common causes of maternal as well as perinatal mortality in the world [176, 177, 178]. The etiology of this gestational disorder is still largely unknown. An imbalance between lipid peroxides, ROS or other (endogenous) toxins on the one hand and detoxicating or scavenging substances on the other may contribute to its etiology [179, 180]. GSH and GSH-related enzymes have been investigated in numerous studies for their possible role in the etiology and prevention of this disease.

In normal pregnancy, plasma thiol levels show a decrease, whereas erythrocyte thiol levels increase as compared to levels in the non-pregnant state, suggesting enhanced free radical activity in normal pregnancy [181]. In accordance to these results, erythrocyte GSH levels are reported to be higher in normotensive pregnant women as compared to GSH levels in non-pregnant women [182]. Conflicting reports have been published on thiols and GSH levels in hypertensive disorders of pregnancy. Wisdom *et al.* have reported that in pregnancy-induced hypertension with or without proteinuria, the erythrocytic thiol levels are not different from those of non-pregnant women [181], whereas plasma thiol and GSH levels are significantly lower [182, 183, 184]. Chen *et al.* have demonstrated a significantly positive correlation between levels of prostaglandins and antioxidant capacity [183]. The erythrocytic GSH levels in women with preeclampsia are significantly lower as compared to levels in normotensive

pregnant controls [184]

Although pregnancy induces a gradual increase in lipid peroxidation products with increasing length of gestation, maternal erythrocyte GPX activity does not increase in pregnancy [185, 186] Plasma and erythrocyte GPX levels even decrease after the 20th and 30th week of pregnancy [187] Recently, we have found a decreased GSH/haemoglobin ratio in patients with preeclampsia or the HELLP syndrome as compared to ratios in normotensive pregnancy [188, *Paragraph 5 2*]

In patients with pregnancy induced hypertension platelet and plasma GPX activities are significantly higher than those of normotensive pregnant women [189, 190] Patients with severe or superimposed preeclampsia even show significantly higher erythrocyte and plasma GPX activities as compared to those with mild preeclampsia [189 190] High maternal erythrocyte and plasma GPX activities are associated with fetal growth retardation and neonatal asphyxia [190] Maternal serum selenium concentrations are not different in preeclampsia as compared to normotensive pregnancy, stressing the importance of factors other than selenium to the activity of GPX [191] It is interesting to note that in contrast to maternal erythrocytes, erythrocytic GPX activity is significantly lower in umbilical cord blood in hypertensive pregnancy as compared to normotensive pregnancy [192]

An enhanced maternal GPX activity and decreased GSH levels in hypertensive disorders of pregnancy suggest a compensatory mechanism, implicating an enhanced turnover of GSH in response to high lipid peroxide or ROS levels A potential new therapy for hypertensive disorders of pregnancy may therefore be the supplementation of GSH or GSH precursors For instance, N-acetylcysteine, a precursor of glutathione, has been shown to enhance thiol levels in erythrocytes exposed to oxidizing agents, as well as unexposed erythrocytes [193] Clinical studies have reported that S-nitrosoglutathione, a platelet-specific nitric oxide donor, and a potent vasodilator and inhibitor of platelet aggregation, lowers maternal arterial bloodpressure, and reduces platelet activation and uterine artery resistance without affecting fetal Doppler indices [194] In addition, a pilot study has shown that the administration of S-nitrosoglutathione improves clinical symptoms in a patient with HELLP syndrome [195]

Few studies on decidual tissue in hypertensive disorders of pregnancy have been published An enhanced lipid peroxidation may be involved in decidual foam cell formation, and in the pathogenesis of preeclampsia [196] Preeclampsia is associated with a change of the prostaglandin / thromboxane balance in favour of thromboxane formation Human decidua mainly produces thromboxane B_2 (TxB_2), however after addition of reduced glutathione, prostaglandin E_2 (PGE_2) becomes the main product

[197] Cytosols from decidua vera and placenta are most effective in stimulating synthesis of PGE_2. Reduced GSH is able to increase the biosynthesis of PGE_2 at the expense of other prostaglandins (PG's), both in the presence or absence of these cytosols [198]. GSH can inhibit lipid peroxidation via membrane bound GPX [4]. In the light of these results it is interesting that enhanced decidual GSH levels in preeclampsia were demonstrated in a recent study [*Paragraph 5.1*]. Decidua of normal and preeclamptic pregnancies demonstrates much higher GSH levels than any other human tissue studied [199, 200, 201]. An increase in decidual tissue GSH concentration may point to a preventive mechanism against excessive lipid peroxidation, and may even enhance prostaglandin production. Significantly higher SeGPX activities, as found in decidual tissue of preeclamptic pregnancies as compared to decidual tissue in normotensive pregnancies, may also act as a compensatory mechanism [*Paragraph 5.1*]. By contrast, no difference in decidual GST activity has been found between preeclampsia and normotensive pregnancy [*Paragraph 5.1*].

Placental GSH levels are reported to be higher in preeclamptic pregnancy as compared to placental GSH levels in normotensive pregnancy [202, *Paragraph 5.1*]. As placental cytosols show to be most effective in stimulating PGE_2 biosynthesis, and reduced GSH was found to increase the biosynthesis of PGE_2 at the expense of other PG's [198], the high placental GSH levels in preeclampsia may also point to a compensatory mechanism.

With respect to GPX activities in placental tissue of preeclamptic pregnancies conflicting results have been reported, varying from decreased to increased activities. GPX activity [186, 203] as well as messenger RNA levels of GPX [204] are lower in placentas of preeclamptic pregnancies as compared to those in normotensive pregnancies, whereas levels of lipid peroxides are higher [205]. This may lead to an increased stimulation of prostaglandin H synthetase, again resulting in an enhanced production of lipid peroxides and thromboxane [203]. One of the functions of GPX is the inactivation of lipid peroxides, thereby diminishing peroxide mediated stimulation of prostaglandin synthetase. By contrast, in other studies normal placental GPX activities were demonstrated in preeclampsia as compared to normotensive pregnancy [206], as well as significantly higher GPX activities [*Paragraph 5.1*]. Placental GST activity is found not to differ between preeclampsia and normotensive pregnancy [206, *Paragraph 5.1*].

An interesting hypothesis on the etiology of preeclampsia is a possibly low or a unfavourably increased inherent maternal detoxification capacity. A slightly higher incidence of the GSTT1-1 null genotype, resulting in a complete absence of this

detoxificating enzyme, is found in patients with preeclampsia and/or the HELLP syndrome, whereas no difference is found for the incidence of the GSTM1-1 null genotype [Zusterzeel *et al.*, unpublished results] As previously indicated, individuals with a functional GSTT1-1 allele can form mutagenic metabolites from dichloromethane once this compound is conjugated with GSH [74, 207] So an *increased* incidence of the GSTT1-1 null genotype may contribute to the etiology of preeclampsia or the HELLP syndrome. It should be stressed, however, that generally speaking reactions catalyzed by GST lead to detoxification.

Generally speaking, maternal circulating GSH levels seem to be decreased, while GPX levels seem to be increased in hypertensive disorders of pregnancy, suggesting an enhanced detoxificational capacity at the expense of glutathione. Decidual detoxification seems to be enhanced, possibly at the expense of circulating maternal GSH levels, while conflicting results have been published on placental GSH and GSH-related enzymes. Supplementation of glutathione-enhancing agents, such as N-acetylcysteine, may be beneficial in the treatment of these gestational disorders

7.2 GSTs as markers for haemolysis and hepatocellular impairment in hypertensive disorders of pregnancy.

As reported earlier in this review, circulating GST subtypes can act as markers for disease. In two studies it has been demonstrated that plasma GSTA1-1 is a more sensitive and specific parameter for maternal hepatocellular impairment than ALT activity (*Fig 4*) [208, 209, *Paragraph 5.3*]. Recently, hepatocellular impairment in the HELLP syndrome has been demonstrated to be restricted to the mother, without any evidence for neonatal hepatocellular impairment. In this study neonatal hepatocellular impairment has been assessed by means of umbilical cord plasma GSTA1-1 measurements [210, *Paragraph 5.4*].

In hypertensive disorders of pregnancy, haemolysis is well recognized as a major complication [211], and may even be a predictor for fetal and maternal outcome [212] Measurement of serum lactate dehydrogenase (LDH) activity may not accurately reflect haemolysis, because except from erythrocytes, serum LDH may also originate from the cardiac and skeletal muscle, kidney, pancreas or liver Haptoglobin is considered to be a more reliable indicator for haemolysis in hypertensive disorders of pregnancy [213] but concurrent hepatocellular impairment could lead to falsely low levels [214]. Because GSTP1-1 is present in large quantities in erythrocytes [66, 215], measurements of plasma GSTP1-1 are suggested to be a reliable indicator for haemolysis in

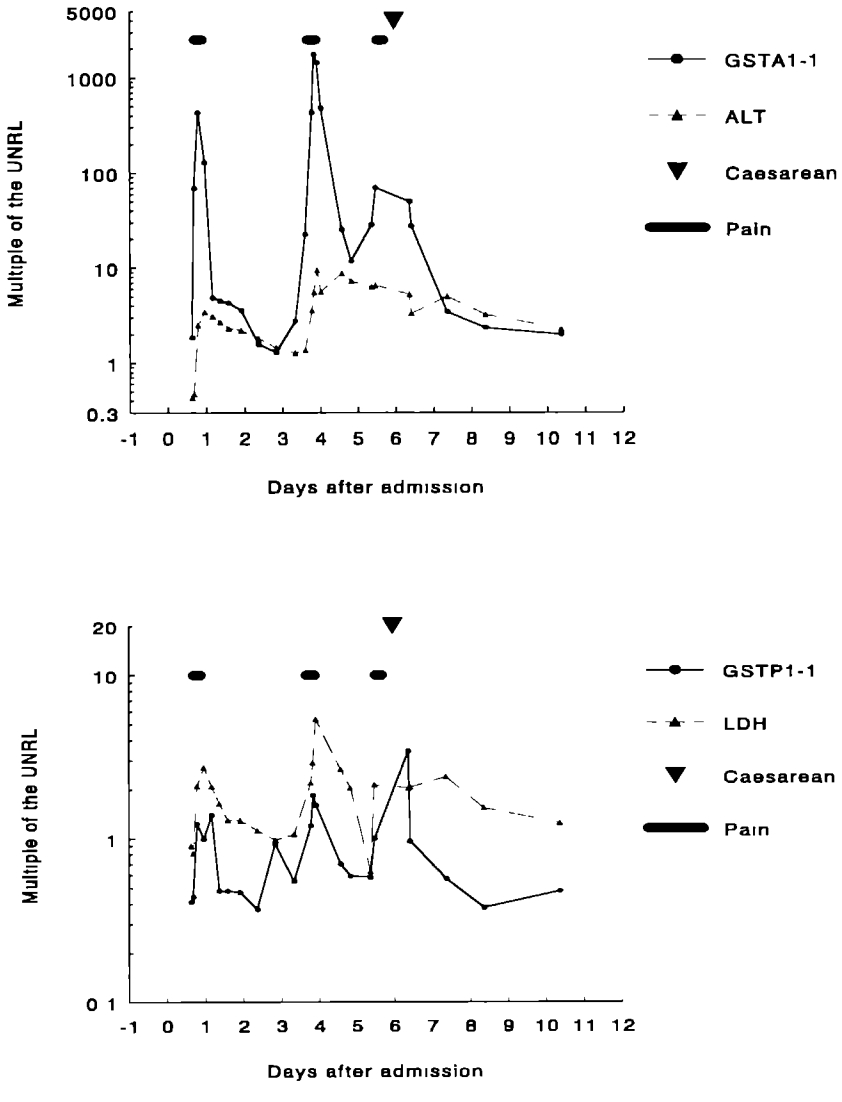

Fig. 4 *Plasma GSTA1-1 / UNRL and serum ALT / UNRL (upper graph); plasma GSTP1-1 /UNRL and serum LDH/UNRL (lower graph) in a HELLP patient, studied longitudinally. (Reprinted with permission from The Lancet), Pain = epigastric pain*

haematological diseases such as paroxysmal nocturnal haemoglobinuria [87] GSTP1-1 levels are indeed found to be significantly elevated in preeclampsia and the HELLP syndrome as compared to normotensive pregnancy, and may be a more reliable indicator of haemolysis in these syndromes *(Fig. 4)* [*Paragraph 5 5*]

In summary, GSTA1-1 seems to be a superior marker for hepatocellular impairment in hypertensive disorders of pregnancy, and GSTP1-1 may be a reliable marker for haemolysis in these multi-system disorders.

8. THE GLUTATHIONE / GLUTATHIONE-RELATED ENZYME SYSTEM IN NEONATAL LIFE.

GSH is an essential component of the pulmonary antioxidant defence system in neonates, and may be of particular importance in the early phase of oxygen exposure. The lungs of the prematurely born infant may be ill-adapted for the protection against high oxygen exposure, due to the immaturity of its antioxidant defence systems [216]. The deficiency of GSH in the lungs of preterm delivered animals may attribute to the increased susceptibility to oxygen-induced pulmonary injury [217] GSH levels in broncho-alveolar lavage fluid, measured in neonates requiring assisted ventilation, have demonstrated the lowest GSH levels in the patients with the most severe airway problems who had required the highest supply of oxygen [218] In newborn rats exposed to 100% oxygen, a rapid and sustained increase in total GSH, primarily due to an increase in reduced glutathione, has been demonstrated. In addition, enhanced activities of GPX and GSH reductase are noted, representing an important protective mechanism against oxidant injury [219]. An improvement in the oxygenation of premature infants with idiopathic respiratory distress syndrome (IRDS) is accompanied by an improvement of the ratio of reduced to oxidized glutathione (GSH redox-ratio), indicating an enhanced ROS inactivating capacity [220] The transition of rats from fetal to neonatal life is associated with a decrease of the GSH redox-ratio in the liver, which can partially be prevented by N-acetylcysteine administration to the mother [221] By contrast, no difference has been demonstrated between erythrocyte GPX activity of babies who suffer from IRDS and matched controls [222] Clinical pharmacological strategies, specifically designed to augment the pulmonary antioxidant defence capacity may become available in the near future [216].

Plasma cysteine levels are much lower in premature infants as compared to plasma cysteine levels in mature infants [223]. In addition, erythrocytes of premature infants show a much lower rate of GSH synthesis from L-methionine (a process dependent on the cystathionase pathway) [223]. In premature infants, the metabolic flow through the

transsulfuration pathway may be insufficient to meet their GSH and cysteine requirements [223]. Higher GSH concentrations are present in neonatal erythrocytes as compared to those in adults. For unknown reasons, higher GSH concentrations are an epiphenomenon in inherited pyrimidine 5'-nucleotidase deficiency [224] GSH levels in umbilical cord blood increase with advancing gestational age [225]

Significantly lower erythrocyte GPX activity and serum selenium concentrations are demonstrated in neonates as compared to adult controls [226]. Yet, Rotilio *et al* found approximately similar GPX activities in neonates and controls [227]. They associate a high superoxide dismutase / GSH peroxidase ratio with haematological symptoms [227]. Erythrocyte GPX activity is found to be lower in prematurely born infants as compared to full-term neonates [228] There is no difference between neonatal venous and arterial umbilical cord whole blood GPX activities and GPX activity was found to be increasing with gestational age and birth weight [229]. In another study, however, whole blood GPX activity is not found to be related to birth weight or gestational age [230].

A deficiency of GSH synthetase is responsible for the disease 5-oxoprolinuria, characterized by haemolysis, metabolic acidosis, defective function of the central nervous system and 5-oxoprolinuria. Biochemical studies confirm that an enzymatic block of the gamma glutamyl cycle leads to a generalized GSH deficiency [231, 232] In a case report on neonatal 5-oxoprolinuria, GSH and GSH synthetase activity are found to be 25% and 5% of control values respectively [233].

Jaundiced newborns with normal glucose-6-phosphatase (G6PD) activity have significantly lower levels of GSH reductase (GSSGR) than non-jaundiced newborns with normal G6PD activity [234]. Erythrocyte GPX deficiency, as demonstrated in a Japanese family is associated with neonatal hyperbilirubinaemia [235] Erythrocyte GPX or GSH deficiency in neonates shows to be more frequently associated with pathologic jaundice than in the absence of such deficiency (12.8% vs. 5 4%, respectively) [236]

GSH levels in umbilical cord blood are lower after asphyxia [225]. The hepatic function of neonates suffering from asphyxia at birth is usually assessed by measuring neonatal venous blood aspartate aminotransferase (AST, EC 2.6.1.1) or alanine aminotransferase (ALT; EC 2.6.1.2) activities. These activities, however, do not rise significantly during the first hours after birth complicated by asphyxia and therefore lack sensitivity in the early postpartum period [237]. The Alpha class GSTs are rapidly released in relatively large amounts into the bloodstream after hepatocellular damage [6]. In asphyxiated newborns, venous plasma GSTB1 levels (GSTB1 being the former name for GSTA1-1) are shown to be a more sensitive indicator for hepatocellular

impairment than serum ALT activities [238]. In addition, arterial umbilical cord plasma GSTA1-1 levels demonstrate an association between hepatocellular and metabolic or hypoxic impairment [239, *Paragraph 6.2*], and prove to be a superior marker for neonatal hepatocellular impairment as compared to the corresponding AST and ALT activities [*Paragraph 6.1*]. Previously, the *in utero* fetal venous umbilical cord GSTB1 concentrations have been shown to be elevated in frankly acidotic human fetus as compared to the concentrations in non-acidotic fetus [240].

As for GSTP1-1, it has been demonstrated that high GSTP1-1 concentrations in cerebrospinal fluid during pediatric meningitis may indicate the severity of inflammation and may thus be of prognostic significance [241]. An aberrant expression of GSTP1-1 is identified in hepatocytes of 14 out of 15 neonates with neonatal biliary atresia and in only two out of 12 cases of neonatal hepatitis, whereas no abnormalities are found for GST Alpha [242].

In summary, a deficiency of GSH in lungs of premature infants may contribute to oxygen-induced pulmonary damage A deficiency of glutathione synthetase is responsible for the disease 5-oxoprolinuria. Neonatal venous GSTA1-1 plasma concentrations is a more valuable marker for asphyxia at birth as compared to AST or ALT concentrations, while umbilical cord GSTA1-1 concentrations may be a valuable marker for early hepatocellular impairment due to the process of birth

9. HORMONAL INFLUENCES ON GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES.

Numerous studies have shown that hormonal status, sex and menstrual phase significantly influence the expression of GSH and GSH-related enzymes. Mouse [243], rat [244], and human class Alpha, Mu, and Pi GSTs are subject to sex-specific variations [9].

The menstrual phase seems to have a significant influence on tissue GSH levels, such as those in breast and fat tissue, with the largest effect in the mid-luteal phase [245] GPX activity in human endometrium has also been shown to vary with the menstrual phase, being highest in the late proliferative and early secretory phase [246]. Amenorrhoic women demonstrate significantly lower erythrocyte GPX activities as compared to GPX activities in eumenorrhoic women, and when oestradiol concentrations are normalized by means of transdermal oestradiol supplementation therapy, erythrocyte GPX activity is also restored. Medroxyprogesterone, by contrast, does not influence erythrocyte GPX activity [247]

Men have demonstrated significantly higher plasma GSTA1-1 concentrations than

women [248, 249]. In women, but not in men, a significant increase of plasma GSTA1-1 levels is noted with age [248, 249] It has recently been demonstrated that the administration of progesterone, but not oestradiol, to healthy postmenopausal women leads to a significant decrease in plasma GSTA1-1 levels yielding premenopausal levels [250, *Chapter 7*]. Interestingly, ovariectomy in mice has resulted in the enhancement of liver GST activities, which can be normalised by oestrogen or progesterone administration [243]. The high plasma GSTA1-1 levels in postmenopausal women may point to an increased hepatocellular leakage of GSTA1-1 from liver tissue of these women or point to a progesterone dependent downregulation of GST expression in the liver.

Men show significantly higher GSTP1-1 plasma levels than women, and a significant increase with age is noted for both sexes [86]. There is no clear explanation for this phenomenon GPX activity is significantly lower in blood of postmenopausal women as compared to GPX activity in premenopausal women [251], and GPX activity in rat endometrial tissue is enhanced by progesterone administration, but decreased by means of oestradiol treatment [246].

Other studies on hormonal dependence of GSH and GSH-related enzymes show that dietary dihydroepiandosterone administration to rats and mice enhances GST activity in the liver [252]. Limited studies have indicated a role for growth hormone and thyroxine in sex-dependent expression of GST Alpha and Mu mRNAs in rat liver [253] The NO donors S-Nitroso-L-acetyl penicillamine (S-NAP) and S-nitrosoglutathione cause a dose-dependent decrease in both oestradiol and progesterone secretion in granulosa-luteal cell cultures [254].

In summary, GPX and GST isoenzymes are under hormonal control. GPX activity seems to be influenced by oestradiol levels, whereas GST isoenzymes are most probably influenced by progesterone levels

10. GLUTATHIONE AND GLUTATHIONE-RELATED ENZYMES AND GYNAECOLOGICAL CANCER.

Humans vary in their ability to metabolize carcinogens and individuals with reduced ability to detoxify endogenous and exogenous carcinogens may be at increased risk for the development of cancer. Conjugation with GSH of electrophilic compounds, catalyzed by GSTs, is an important metabolic pathway for detoxification of mutagens and carcinogens [255]. Numerous studies on the role of GSH and GSH-related enzymes in gynaecological tumours, such as cervical, endometrial, ovarian, uterine and breast cancer, have been published.

Most studies are dedicated to ovarian tumours, probably because ovarian cancer is the most lethal gynaecological malignancy. An enhanced expression of GSTs may be a marker of malignant transformation of ovarian, and cervical cells [166, 256, 257]. Malignant ovarian tumours express higher GST levels as compared to benign tumours [258]. In another study, however, mean GST activity is found to be lower in ovarian cancerous tissue as compared to GST activity in normal and benign tissue, while GST activity is similar in serous, as compared to GST activity in other malignant ovarian tumours [259]. Some studies report high expression of GSTP1-1 to be a predictor for resistance to chemotherapy and a useful marker for poor prognosis in ovarian carcinoma [84, 85]. However, in other studies GST activity [260, 261], GSTP1-1 [261, 262], GST Alpha [261, 262] and GST Mu [261] expression in particular, show no significant correlation with prognosis or survival of patients with ovarian tumours. In addition, no association is found between the expression of GSTs and the response to chemotherapy [263]. Furthermore, no association is found between tumour GSH levels and GST Alpha or GST Mu expression, and the response to single agent carboplatin treatment in primary ovarian cancer [264]. Other studies report that high GST activity in ovarian tumours is associated with a better response to chemotherapy, and survival [265], and that patients with ovarian carcinoma expressing GST, exhibit better survival rates [266]. Administration of cisplatin, adriamycin and cyclophosphamide seem to induce expression of GST [266]. Lower tumour GSH content is associated with lower FIGO stages, and prolonged survival [262]. The ratios of GSH content as well as GSTP1-1 activity in ovarian carcinoma before chemotherapy to corresponding levels after chemotherapy are higher in non-responders as compared to responders, which indicates that GSH and GSTP1-1 are associated with drug resistance in these patients [267].

Conflicting results are reported on the expression of GST in metastatic disease. An independent positive correlation between GSTP1-1 expression in ovarian tumours and subsequent para-aortal lymph node metastasis [257] was noticed, whereas no significant relationship between the expression of GSTP1-1 and the incidence of metastasis is found by Volm *et al.* [268]. Recently, clinical trials were initiated with BSO, which *in vitro* decreases the ability of resistant ovarian carcinoma cells to inactivate platinum compounds and alkylating agents [269]. In a phase I trial of ovarian cancer it is demonstrated that both in normal and tumorous tissue GSH can be depleted after supplementation with BSO [270]. Systemic co-administration of the chemoprotectant GSH in combination with cisplatin to patients with ovarian cancer results in higher attainable cisplatin dosages [271].

Only a few studies on endometrial cancer have been published. Enhanced GPX activities are associated with well differentiated rather than poorly and moderately differentiated endometrial adenocarcinomas [246]. GST activity is reported to be significantly lower in endometrial cancer as compared to normal endometrium in both a Japanese and a Finnish population [272]. In another study an over-expression of GST in endometrial carcinoma is suggested to account partly for the resistance to chemotherapy of this tumour [273] The risk for endometrium cancer is not associated with polymorphisms for GSTM1-1 or GSTT1-1 [274]

An enhanced expression of GST is demonstrated in cervical carcinoma [166], which suggests it to be a marker of malignant transformation of cervical cells. Nevertheless, GST is present in adult cervical squamous epithelia under normal as well as pathological conditions and it is therefore not necessarily linked to carcinogenesis. GSTs are therefore not likely to be of any use as markers for cervical epithelial neoplasia [275] The GSH content of cervical squamous cell carcinoma is twofold higher than that of normal cervical tissue [276]. The tumour margin shows a higher GSH content than the centre of the tumour [276]. Enhanced erythrocyte GSH and GSH reductase activities are associated with the severity of cervical dysplasia [277]. Changes in erythrocyte GSH-related indices, together with the histopathological diagnosis may have the potential to distinguish low- and high grade cervical dysplastic lesions from one another [277] The presence of GSTM1-1 or the GSTT1-1 null genotype does not appear to influence the susceptibility to cervical intra-epithelial neoplasia or squamous cell carcinoma [278, 279].

Several studies have been published concerning breast carcinoma and GSH and GSH-related enzymes. It is reported that gamma glutamyl transpeptidase (γGT) expression is lost, depending on the degree of malignancy in breast cancer [280]. Interestingly, GSH content in breast and adipose tissue is dependent on the menstrual cycle: increasing in the luteal phase and being highest in the mid-luteal phase [245]. Breast tumours do not express higher levels of GSTs as compared to adjacent normal breast tissue [7, 10], although a more recent study has demonstrated enhanced GST levels in tumorous breast tissue as compared to adjacent normal tissue [281], GST Mu [282] and GSTP1-1 [282] in particular. An enhanced GST expression may be involved in chemotherapy resistance of breast tumours [283], but many other studies do not find a correlation between GST Alpha, Mu or Pi content of the primary tumour and the response or duration of the response to chemotherapy [284, 285]. Additionally, GST Mu deficiency does not seem to be involved in the etiology of breast cancer [284] Recent studies, however, have demonstrated high levels of GSH, GST and GPX to be associated with

favourable clinical characteristics and prognosis, whereas low levels of GSH and GST activity are associated with more aggressive or more advanced disease in breast cancer patients [286]

The presence of the GSTP1b allele seems strongly associated with testicular cancer, whereas in prostate cancer a significantly lower frequency of the GSTP1a allele has been observed [287] Testicular germ cell tumours show an altered isoenzyme pattern and the decreased GST activity may play a role in the high inherent drug sensitivity of human testicular germ cell tumours [288]

Cytotoxic chemotherapy in gynaecological cancer do not change the activity of serum GPX [289] and patients with gynaecological cancer (uterine, ovarian or vulvar) have lower serum GPX activities as compared to healthy control women [290]

In summary, in early studies, GSTP1-1 plasma levels seem to be a reliable marker for gynaecological cancer and high GST expression, especially in ovarian tumours seems to be indicative of poor prognosis, probably due to chemotherapy resistance In many other studies however, these results have not been confirmed.

11 MISCELLANEOUS.

In areas with heavy radioactive exposure, such as the Ukrain areas, placental samples have been shown to contain significantly lower GST and GSH-reductase activities, and lower levels of thiols as compared to samples in non polluted areas [291] Maternal cigarette smoking has been found not to affect placental GST activity, but a positive correlation between placental GST and aromatase activity has been demonstrated [292]

Erythrocyte GPX activities are shown to be increased in adult Down syndrome [293] A high incidence of the GSTM1-1 null genotype has been found in several multifactorial diseases, such as endometriosis, favouring the contribution of environmental toxins in the pathogenesis of this disease [294, 295]

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CHAPTER 3

Glutathione S-transferases in human ovarian follicular fluid

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ABSTRACT.

Objective: To study the levels of glutathione S-transferase Alpha 1-1 (GSTA1 1) and glutathione S-transferase Pi 1-1 (GSTP1-1) in human preovulatory ovarian follicular fluid (FF) and pooled granulosa and cumulus cells

Design: The relation of GSTA1-1 and GSTP1-1 with progesterone (P) and 17β oestradiol (17β-E_2) concentrations were studied.

Setting: The Department of Obstetrics and Gynaecology, the Department of Gastroenterology and the Laboratory of Endocrinology and Reproduction of the University Hospital St Radboud in Nijmegen, The Netherlands

Patient(s): Infertile women participating in an in-vitro fertilization programme

Result(s): Detectable amounts of GSTA1-1 and GSTP1-1 were found in ovarian follicular fluid and pooled cumulus and granulosa cells. Concentrations of GSTA1-1 were always much higher than those of GSTP1-1. Both ovarian FF concentrations of GSTA1-1 and GSTP1-1 did not correlate with ovarian FF concentrations of 17β-E_2 and P

Conclusion(s): The high FF concentrations of GSTP1-1 and especially of GSTA1-1 suggest that these enzymes may play an important role in the detoxification processes in the follicles The lack of correlation between follicular P and 17β-E_2 and GSTA1-1 and GSTP1-1 indicates that both enzymes presumably are not present as a result of the high steroid levels

INTRODUCTION.

Follicular fluid (FF) is formed by a gradual influx of fluid derived from the blood and by compounds synthesized and secreted by theca and granulosa cells. Therefore, its composition reflects both the plasma composition and the secretory activity of theca and granulosa cells [1]. Follicular fluid in humans and animals contains much higher concentrations of both progesterone (P) and 17β-oestradiol (17β-E_2) than does blood [2]

The glutathione S-transferases (GST, EC 2.5.1.18) are a multigene family of enzymes that catalyze the nucleophilic addition of glutathione to the electrophilic centres of a wide variety of xenobiotics They also serve as transport proteins for a broad range of lipophilic compounds, such as bilirubin, bile acids, and steroid hormones Glutathione S-transferase Alpha seems to be associated closely with the glutathione-dependent Δ5-3-ketosteroid isomerase which catalyzes the conversion of pregnenolone

to P and of dehydroepiandrosterone to androstenedione, together with 3β hydroxy steroid dehydrogenase [3] On the basis of their structural, physico-chemical, enzymatic, and immunological properties, the family of enzymes is divided into four classes Alpha, Mu, Pi and Theta, each of which consists of one or more isoforms [4 5]

Glutathione S transferases have been shown to be present in ovarian luteal tissue in pig [6] and rat ovaries [3] as well as in normal human ovarian tissue [7 8] Immunohistochemical staining for GST Alpha and GST Pi in human ovaries revealed that granulosa cells in graafian follicles as well as in corpora lutea, but not in primordial, primary and atretic follicles stained for GST Alpha and to a lesser extent for GST Pi Glutathione S-transferase Alpha strikingly parallels sites of steroid production

To our knowledge the presence of GSTs in human FF has never been reported Therefore, we initiated this study to determine the presence and concentrations of glutathione S-transferase Alpha 1 1 (GSTA1-1) and glutathione S transferase Pi 1 1 (GSTP1-1), isoforms of the class Alpha and class Pi GSTs, respectively, in human preovulatory FF

Further, we determined the correlation of both GSTA1 1 and GSTP1 1 with ovarian FF concentrations of 17β-oestradiol (17β E_2) and progesterone (P) to investigate the putative role of these enzymes in the detoxification or transport of the very high steroid levels We also determined GST concentrations in pooled granulosa and cumulus cells in order to find a possible origin for GSTA1-1 and GSTP1 1 in the FF

MATERIALS AND METHODS.

Study population

After informed consent was given, ovarian FF samples were obtained from 10 women participating in an in vitro fertilization (IVF) programme The experimental protocol was approved by the Medical Ethical Review Committee of the University Hospital Nijmegen Multiple follicular development was induced by human menopausal gonadotropins (hMG, Humegon, Organon Oss, the Netherlands) as described previously [9] A gonadotropin-releasing hormone agonist (GnRH, 1 mg, Lucrin, Abbott, Amstelveen, the Netherlands) was administered subcutaneously from day 21 of the preceding cycle until the evening of human chorionic gonadotropin (hCG, Pregnyl, Organon, Oss, The Netherlands) injection The dosage of hMG was based on the serum E_2 concentration and varied between 150 and 300 IU/d

As soon as the serum E_2 concentration reached about 900 pmol/L per follicle, and the diameter of the follicle reached >15 mm, 10,000 IU of hCG was injected intramuscularly. Follicular fluid was aspirated by transvaginal puncture using ultrasound guidance and fluid from three follicles was obtained from each patient. The FF was centrifuged separately for 10 minutes at 3,000 x *g* to remove red blood cells. The supernatant was frozen and stored at - 20°C until analysis. The FF was examined separately for GSTA1-1 and GSTP1-1 and for their corresponding 17β-E_2 and P concentrations. From each patient, the follicle with the highest 17β-E_2 concentration was used for statistical evaluation.

Forty oocyte-corona-cumulus cell complexes were isolated from follicular aspirates. They were visualized under Leitz (Dialux EB 20, Wetzlar, Germany) optics to establish the morphology. Only mature cumulus cell complexes were used (10). Cumulus cells from all complexes were placed separately in 2 mL of human tubal fluid (HTF) medium and were washed three times in the same volume of HTF [9]. Thereafter the cumulus cells were pooled, placed in 10 mL of HTF and centrifuged at 300 x *g* for 10 minutes.

The granulosa cells were isolated from clear FF aspirates that were visually free from blood. Naturally, the aspirated cells contained red blood cells. These were removed almost completely by allowing them to settle in 100-mm Petri dishes (Falcon 1001) containing 5 mL of HTF for 10 minutes, whereafter the granulosa cells were transferred using a Pasteur pipette to a new Petri dish with fresh HTF.

This procedure was performed three times and then the cells were pooled, placed in 10 mL of HTF and centrifuged at 300 x *g* for 10 minutes. The cells were homogenized in 10 volumes of buffer (250 mM of sucrose, 20 mM of Tris-HCl, and 1 mM of dithiothreitol, pH 7,4) with 10 strokes in small glass-glass tissue grinders on ice. The homogenates were centrifuged at 150,000 x *g* at 4° C for 1 hour. The supernatants were frozen at - 70°C until analysis.

Blood plasma concentrations of GSTA1-1 and GSTP1-1 were also determined in the blood plasma samples of 20 patients taken during follicle puncture. Antecubital venous blood samples were taken in the sitting position after 5 minutes of rest in 4-mL ethylenediaminetetraacetic acid tubes (no 606601, Becton and Dickinson, Grenoble, France). Blood was centrifuged at 3,000 x *g* for 10 minutes at 20°C. Plasma was removed and stored at -20°C until analysis.

Assays.

Glutathione S transferase Alpha 1-1 and GSTP1-1 levels were determined using

recently developed ELISAs [11, 12]. Briefly, microtiter plates were coated overnight with purified monoclonal antibody. One hundred microliters of standard (0.08-20 μg/L for GSTA1-1; 0.4-100 μg/L for GSTP1-1) or 1:1 diluted plasma samples or 1:1 and 1:100 diluted FF samples were added to the wells and incubated overnight. The next day, the plates were washed and incubated with rabbit anti-GSTA1-1 or anti-GSTP1-1 antiserum. They were washed again and were incubated with horseradish peroxidase-labelled swine anti-rabbit antigen. After a final wash, the plates were stained with *o*-phenylenediamine-H_2O_2.

The GSTA1-1 assay has a minimum detectable concentration of 0.04 μg/L and intra- and interassay coefficients of variation of 2.5% and 7.3%, respectively. In the case of GSTP1-1 these values are 0.4 μg/L, 5.8%, and 10.9% respectively. Dilutions of these FF samples were parallel to the standard curves and addition of 3,700 nmol/L 17β-E_2 or 1600 nmol/L of P had no effect on the standard curves of the GSTA1-1 or GSTP1-1 assay. Determinations of P and 17β-E_2 concentrations were performed after diethyl ether extraction and Sephadex LH-20 chromatography (Pharmacia, Woerden, the Netherlands) in slightly modified RIAs as described previously [13]. The minimum detectable concentrations of the assays were 10 pmol/L for 17β-E_2 and 1.3 nmol/L for P. Intra-assay and interassay coefficients of variation were 5.4% and 8.4%, respectively, for 17β-E_2 and 5.2% and 7.3%, respectively, for P. Protein concentrations in cumulus and granulosa cells were assayed in duplicate by the method of Lowry *et al.* [14] using bovine serum albumin as a standard.

Statistics.

Correlations between FF 17β-E_2, P, and GSTA1-1 and GSTP1-1 were computed using Spearman's rank correlation test.

RESULTS.

Follicular GSTA1-1 and GSTP1-1 were detectable in FF of all patients studied. Their concentrations as well as those of P and 17β-E_2 are presented in Table 1. The concentrations of GSTA1-1 were approximately 30 times higher than those of GSTP1-1. Glutathione S-transferase Alpha 1-1 and GSTP1-1 also were detected in pooled cumulus and granulosa cells (Table 2). Again, the concentrations of GSTA1-1 were much higher than those of GSTP1-1 (17 and 36 times, respectively). In addition, the enzyme concentrations of granulosa cells were much higher than those of cumulus

cells

Neither follicular GSTA1-1 nor GSTP1-1 concentrations were correlated significantly with follicular P (r = 0 10, P > 0 1 and r = 0 14, P > 0 1, respectively) or 17β-E_2 (r = 0 15, P > 0 1 and r = 0 16, P > 0 1, respectively) concentrations Follicular GSTA1-1 concentrations correlated significantly with GSTP1-1 concentrations (r = 0 93, P = 0 0001) The median concentrations of GSTA1 1 and GSTP1-1 in the blood plasma of 20 patients sampled concurrently at the moment of follicle puncture

Table 1 GSTA1 1, GSTP1-1, P and 17β-E_2 in Human Preovulatory FF

Patient no	*GSTA1 1 (μg/L)*	*GSTP1-1 (μg/L)*	*P (nmol/L)*	*17β-E_2 (nmol/L)*
1	252	17	44	5 8
2	582	29	56	3 9
3	468	17	41	4 4
4	485	21	56	1 0
5	495	17	54	3 3
6	212	15	46	2 6
7	3509	140	13	2 7
8	253	17	27	1 4
9	182	13	33	1 8
10	774	24	44	2 6
Median (range)	477 (182 - 3509)	17 (13 - 140)	44 (13 - 56)	2 7 (1 0 - 5 8)

Table 2 GSTA1 1 and GSTP1-1 in pooled cumulus and granulosa cells

Tissue	*GSTA1-1 (μg/mg protein)*	*GSTP1-1 (μg/mg protein)*
Cumulus	1 42	0 082
Granulosa	12 84	0 35

were 1 71 μg/L (range 0 95 3 57 μg/L) and 6 30 μg/L (range 5 4 - 104 μg/L), respectively These plasma concentrations fell within the normal reference ranges for healthy women of comparable age [11 12]

Discussion.

This study shows that GSTA1 1 and GSTP1-1 are present in considerable amounts in human ovarian FF Glutathione S-transferase Alpha 1 1 is present in far higher concentrations than GSTP1-1 in both FF and pooled cumulus and granulosa cells Median concentrations of both enzymes are higher in follicles of patients undergoing IVF than in their blood plasma The level of GSTA1 1 especially is far above the blood plasma level, indicating a less important role for the influx of this enzyme from blood Remarkably, the concentration of GSTA1 1 in FF as compared to that in blood plasma (FF/plasma ratio ± 280) is much more pronounced than that of GSTP1-1 (ratio ± 2 7) This finding is in concordance with the much higher concentrations of GSTA1 1 in both granulosa and cumulus cells as compared to the concentrations of GSTP1-1

Using immunohistochemical techniques, Rahilly *et al* [8] have demonstrated that GST Alpha is localized to the steroid-producing cells We found that GST concentrations are higher in granulosa cells than in cumulus cells (Table 2) Therefore, it is conceivable that the granulosa cells are the main source of the follicular enzyme concentrations

The functions of GSTA1 1 and GSTP1 1 in ovarian physiology remains unclear Glutathione S-transferase Alpha is associated closely with the glutathione dependent Δ5-3 ketosteroid isomerase which catalyzes the conversion of pregnenolone to P and dehydroepiandrosteron to androstenedione, together with 3β hydroxysteroid dehydrogenase [3] Glutathione S transferase Alpha expression was found immunohistochemically in human granulosa cells during the second half of the preovulatory phase and in corpus luteum tissue, correlating well with sites of Δ5 3 ketosteroid isomerase activity [8]

Glutathione S transferase Alpha also was found in cultured pig granulosa cells In these cells GST Alpha concentrations increased after stimulation by gonadotropins, in association with the production of P [6] However, two studies indicated the independence of GST activity from hormonal variations, whereas cytochrome P 450 dependent hydroxylases seemed to fluctuate with hormonal variations [15, 16]

The lack of any correlation between follicular GSTs and steroid hormones in the same preovulatory phase in women participating in an IVF programme as found in our

study, could point to a role for GSTs as detoxifying enzymes that protect the ovum from reactive oxygen species and xenobiotics Reactive oxygen species may be released in connection with follicle rupture, because inhibition of oxygen free radicals leads to inhibition of ovulation [17] Reactive oxygen species are thought to play a role in oocyte maturation [18] Polychlorinated biphenyls [19, 20] and hexachlorobenzene (HCB) [21], both notorious xenobiotics, have been demonstrated in FF of patients undergoing IVF and are known to induce GST in tissues other than the ovaries [22, 23]

In conclusion, high concentrations of GSTA1-1 and of GSTP1-1 were found in the FF as well as the cumulus and especially the granulosa cells suggesting that these enzymes may play a role in detoxification processes in the follicular micro-environment.

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CHAPTER 4

Localization of glutathione S-transferase Alpha and Pi in human embryonic tissues at 8 weeks' gestational age

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ABSTRACT.

Glutathione S-transferases (GST) are a family of enzymes involved in the detoxification of xenobiotics In humans, GST are divided into four different classes, Alpha, Mu, Pi and Theta, with partly overlapping substrate specificity and a tissue-specific expression pattern. We studied the cellular distribution of GST Alpha and Pi in a variety of human embryonic organs, obtained from an extra-uterine monozygotic twin pregnancy at 8 weeks' gestational age. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Three 4 μm thick sections were used, one for routine haematein and eosin staining, the others for immunohistochemical determination using monoclonal and polyclonal antibodies against GST Alpha and Pi, respectively. Both GST Alpha and Pi were present in hepatocytes, gastrointestinal epithelium, adrenal gland medulla, and tela chorioidea in the telencephalon. GST Pi, but not Alpha was found in the epithelium of pancreatic and pulmonary glands, trachea, nephrons and urinary collecting ducts, as well as in the pia mater of the telencephalon and in developing nerve tissue in the gastrointestinal muscularis mucosae In summary, we have demonstrated that immunoreactive protein for both GST Alpha and Pi is expressed in the human embryo at 8 weeks' gestational age The early expression of GST Alpha and Pi in the epithelia of the urinary and digestive tracts and respiratory system supports the importance of GST in the detoxification of potentially toxic or carcinogenic compounds Our results suggest that the embryo itself is capable of detoxifying noxious compounds that are generated intracellularly or that cross the trophoblastic tissue

INTRODUCTION.

Glutathione S-transferases (GST; EC 2.5.1.18) are a supergene family of dimeric enzymes that catalyze the nucleophilic attack of the sulphur atom of the tripeptide glutathione on the electrophilic centres of a seemingly endless variety of chemical compounds. Since most reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, GST play a critical role in the detoxification of xenobiotics anc carcinogens [1, 2, 3, 4]. Mammalian GST isoenzymes, which are mainly present in the cytosol, have been grouped into four classes based upon primary structure, catalytical properties, immunochemical cross-reactivity and N-terminal amino acid sequences, namely Alpha, Mu, Pi and Theta [2, 4] The isoenzymes of these classes have a partly overlapping substrate specificity and a

tissue specific expression; GST Alpha and Mu predominate in the adult liver and small intestine, whereas GST Pi is mainly found in erythrocytes, kidney, placenta and fetal liver [2, 5] High GST enzyme activity may result in an efficient elimination of carcinogens and ultimately lead to cancer prevention. We recently demonstrated an inverse relation between GST enzyme activity and the incidence of gastrointestinal malignancies, high GST activity in organs with low tumour incidence (liver) and low activity at sites with high tumour incidence (colon) [6]. The presence and distribution of GST isoenzymes in human adult tissues and numerous tumour cell lines have been actively studied [7], for review see Beckett and Hayes [1] and Hayes and Pulford [2].

The analysis of GST isoenzyme distribution in human embryonic tissues may provide insight into the expression of these important detoxification isoenzymes and ultimately may lead to a better understanding of their role in protecting cells from cytotoxic agents Since some drugs and chemicals may cross the placenta and accumulate in fetal tissues [8], a wide distribution of GST may be important in detoxification Only a few reports have been published on the tissue distribution of GST isoenzymes in fetal liver, kidney and lung [9, 10] at 12 weeks' gestational age, but no data have been published concerning expression earlier in development as well as in other tissues, such as the gastrointestinal tract. We now studied the expression of GST Alpha and GST Pi in a variety of embryonic organs, obtained from an ectopic monozygotic twin pregnancy at 8 weeks' gestational age.

MATERIALS AND METHODS.

Tissue samples.

Specimens of male embryonic monozygotic twins were obtained from the Department of Obstetrics and Gynaecology of the University Hospital St Radboud, Nijmegen, the Netherlands, after informed consent from the parents. Pregnancy was induced by an in-vitro fertilization procedure in which two zygotes were replaced in the uterine cavity The woman was admitted for abdominal pain at 8 weeks' gestational age and ultrasound examination showed a vital singleton intra-uterine pregnancy as well as a vital ectopic twin pregnancy. Gestational age was confirmed by ultrasound examination. The ectopic pregnancy was localized at the left ovary and was subsequently removed in total at abdominal surgery. Macroscopic examination showed an intact, mono-chorionic, mono-amniotic embryonic sac, containing two male embryos at Carnegie stage 23 [11] both with a crown-rump length (CRL) of 31 mm Embryonic stomach, small intestine, liver, pancreas,

Table I Immunoreactivity of GST Alpha and Pi in human embryonic tissues and in trophoblastic tissue

Tissue	Cell type	GST Alpha	GST Pi
Stomach	Epithelium	+	+ +
(Fig 1a/1b)	Developing nerves	-	±
	Connective tissue	-	-
Small Intestine	Epithelium	+	+ +
	Developing nerves	-	±
	Connective tissue	-	-
Liver	Hepatocytes	+ +	+ +
(Fig 1c/1d)	Haematopoietic cells	-	-
Pancreas	Tubular epithelium	-	+
	Connective tissue	-	-
Mesonephros	Collecting tube epithelium		+
	Connective tissue		±
Metanephros	Bowman's capsule	-	+
(Fig 1e/1f)	Tubular epithelium	-	+
	Connective tissue	-	-
1° Urinary duct	Epithelium	-	+ +
(Fig 2a/2b)	Connective tissue		-
2° Urinary duct	Epithelium	±	+
	Connective tissue	-	
Adrenal gland	Cortex	-	±
	Medulla	±	±
Trachea	Epithelium		+ +
(Fig 2c/2d)	Chondrocytes		+
	Connective tissue	-	-
Lung	Epithelium	-	+
(Fig 2e/2f)	Developing nerves	-	+
	Connective tissue	-	±
Telencephalon	Tela chorioidea	±	+ +
	Pia mater	-	+ +
	Ependyma cells	-	-
Trophoblastic tissue	Cytotrophoblast cells	±	+ +
	Syncytiotrophoblast cells	-	
	Hoffbauer cells	-	±
	Decidua basalis	-	+ +

Tissues from an extra uterine monozygotic twin pregnancy were immunostained for GST Alpha and Pi as described in Materials and Methods For a detailed description of staining patterns, see the Results section - negative, ± weakly stained, + moderately stained, + + strongly stained

kidney, adrenal gland, trachea, lung, and brain were excized and immediately fixed in 4% paraformaldehyde. In addition a piece of trophoblastic tissue was fixed. After dehydration in graded ethanol the tissues were embedded in paraffin.

Immunohistochemical staining.

From each specimen three 4 μm thick slices were used: one for standard haematein and eosin staining and two for immunohistochemical determination of GST Alpha and GST Pi as described previously [12]. In short, sections were dewaxed in xylol, rehydrated in graded ethanol and immersed in methanol (Merck, Darmstadt, Germany) with 2% hydrogen peroxide (Merck) for 10 minutes to block endogenous peroxidase activity. Subsequently, the sections were preincubated with phosphate-buffered saline (PBS) containing 4% bovine serum albumin (BSA, Boehringer, Mannheim, Germany) and 0.1% Triton X-100 (BDH Chemicals Ltd., Poole, England) (buffer A) to block nonspecific binding. The slides were incubated overnight in a humid environment at 4°C with either primary mouse monoclonal antibody against GST class Alpha (1A11) [13], diluted 1:5,000 in buffer A or rabbit polyclonal antibody against GST class Pi (Biotrin International, Dublin, Ireland) diluted 1:1750 in Buffer A. Class Alpha antibody reacts with GSTA1-1, GSTA1-2 and GSTA2-2. After washing three times 5 min in PBS the slides were subsequently incubated with either peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) diluted 1:100 in buffer A or peroxidase-conjugated swine anti-rabbit immunoglobulin (Dakopatts) diluted 1:40 in buffer A for 45 minutes at room temperature. After three 5 minute washings in PBS staining was performed using 0.1% 3,3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO, USA) in PBS containing 0.01% hydrogen peroxide as peroxidase substrate (3 min). To enhance this staining, slides were incubated in 0.5% $CuSO_4$ for 5 minutes. This step was preceded and followed by a washing step of 10 minutes under running tap water. The sections were counterstained with haematein and examined by light microscopy. Human liver and colon tissues were used as positive controls for GST Alpha and Pi, respectively. Omission of primary antibodies and the use of pre-immune serum served as negative controls.

Results.

Table I shows the qualitative scores for both GST Alpha and Pi staining in all tissues examined.

Stomach and small intestine.

In the stomach and small intestine the epithelial cells were stained for GST Alpha and Pi (Fig. 1a and 1b, respectively). The staining was mainly cytoplasmic It was not possible to discriminate between the different cell types in the gastric or the small intestinal epithelium at this stage of embryonic development. Developing nerve tissue in the muscularis mucosae was weakly stained for GST Pi, but not for GST Alpha. Nuclei in the epithelium as well as the connective tissue and muscularis mucosae were unstained with both antibodies

Liver.

Liver tissue consisted of hepatocytes arranged in double cords together with a large number of haematopoietic cells in and around the sinusoids. Developing ducts were not visible GST Alpha and Pi immunoreactivity was present in most hepatocytes (Fig. 1c and 1d, respectively) Staining was heterogeneous, with some cells staining strongly and others moderately or weakly Generally speaking, hepatocytes around the developing central veins were strongly stained. Staining was mainly cytoplasmic, although in some cells nuclei (but not nucleoli) stained as well.

Pancreas.

Pancreatic tissue did not stain for GST Alpha. Cytoplasmic staining for GST Pi was seen in tubular epithelium of glandular pancreatic tissue, whereas connective tissue was negative.

Kidney and urinary tract.

Sections of embryonic renal tissues showed a primitive renal architecture with nephrons at various stages of development In early metanephric kidney, distal ends of newly formed collecting tubules were surrounded by condensations of cells. These form spheres that will fold into S-shaped bodies, one end forming the primitive Bowman's capsule and glomerulus, the other fusing with the elongating collecting tubule. S-shaped bodies will lengthen and later on in gestation differentiate into the distal and proximal tubule and loop of Henle Immunostaining for GST Pi (Fig 1f) resulted in moderate staining of the nephric tissue; approximately 50% of the epithelial cells in the collecting tube were stained. In the

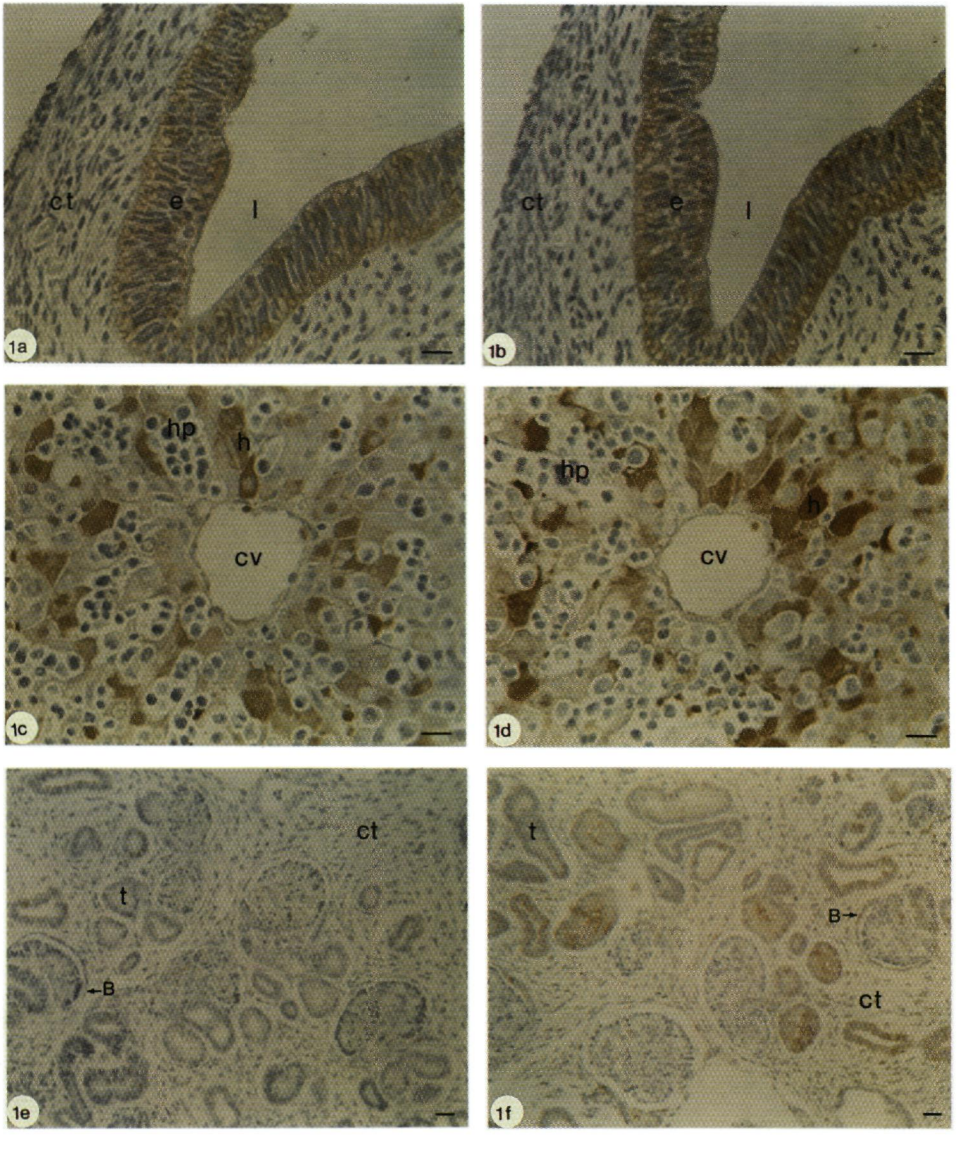

Fig. 1. *Localization of GST Alpha (a,c,e) and GST Pi (b,d,f) in small intestine (a,b), liver (c,d) and nephric tissue (e,f). B, Bowman's capsule; ct, connective tissue; cv, central vein; e, epithelium; h, hepatocyte; hp, haematopoietic cell; l, lumen; t, tubule. Bars indicate 4 μm.*

connective tissue some cells showed a very weak cytoplasmic staining No GST Alpha immunoreactivity was found in both mesonephros and metanephros (Fig 1e) In the latter tissue immunostaining for GST Pi resulted in weak immunostaining of the Bowman's capsule and tubules, whereas glomeruli remained negative The primary urinary duct did not contain GST Alpha immunoreactivity (Fig 2a), whereas with the GST Pi antibody the cytoplasm of many epithelial cells stained strongly (Fig. 2b) The secondary urinary duct showed a very weak cytoplasmic staining in the epithelial cells with the GST Alpha antibody With the GST Pi antibody, on the other hand, approximately 50% of the cells were stained strongly This staining was only cytoplasmic

Adrenal gland.

The cortex did not show immunoreactivity for GST Alpha, whereas in the medulla approximately 5% of the cells showed weak cytoplasmic staining Staining for GST Pi resulted in a weak staining in the cortex, as well as in the medulla. In the medulla roughly the same cells were stained as with the GST Alpha antibody, but the signal was stronger

Trachea.

In the trachea, no immunoreactivity for GST Alpha was demonstrated (Fig 2c) Immunostaining for GST Pi resulted in a very strong staining of the epithelium, whereas only a few chondrocytes were positive (Fig 2d)

Lung in the glandular phase

The pulmonary tissue studied did not contain GST Alpha immunoreactivity (Fig 2e) The GST Pi antibody (Fig 2f) showed a moderate staining in the cytoplasm of cuboidal epithelial cells In addition the mesothelium and most of the cells in the connective tissue were weakly stained This staining was only cytoplasmic Smooth muscle cells surrounding the large ducts were negative, although developing nerve tissue showed moderate immunoreactivity Arteries and arterioles were not stained

Telencephalon.

GST Alpha immunoreactivity was confined to a weak staining of epithelial cells in the tela chorioidea With the GST Pi antibody, these cells were stained strongly, as were epithelial

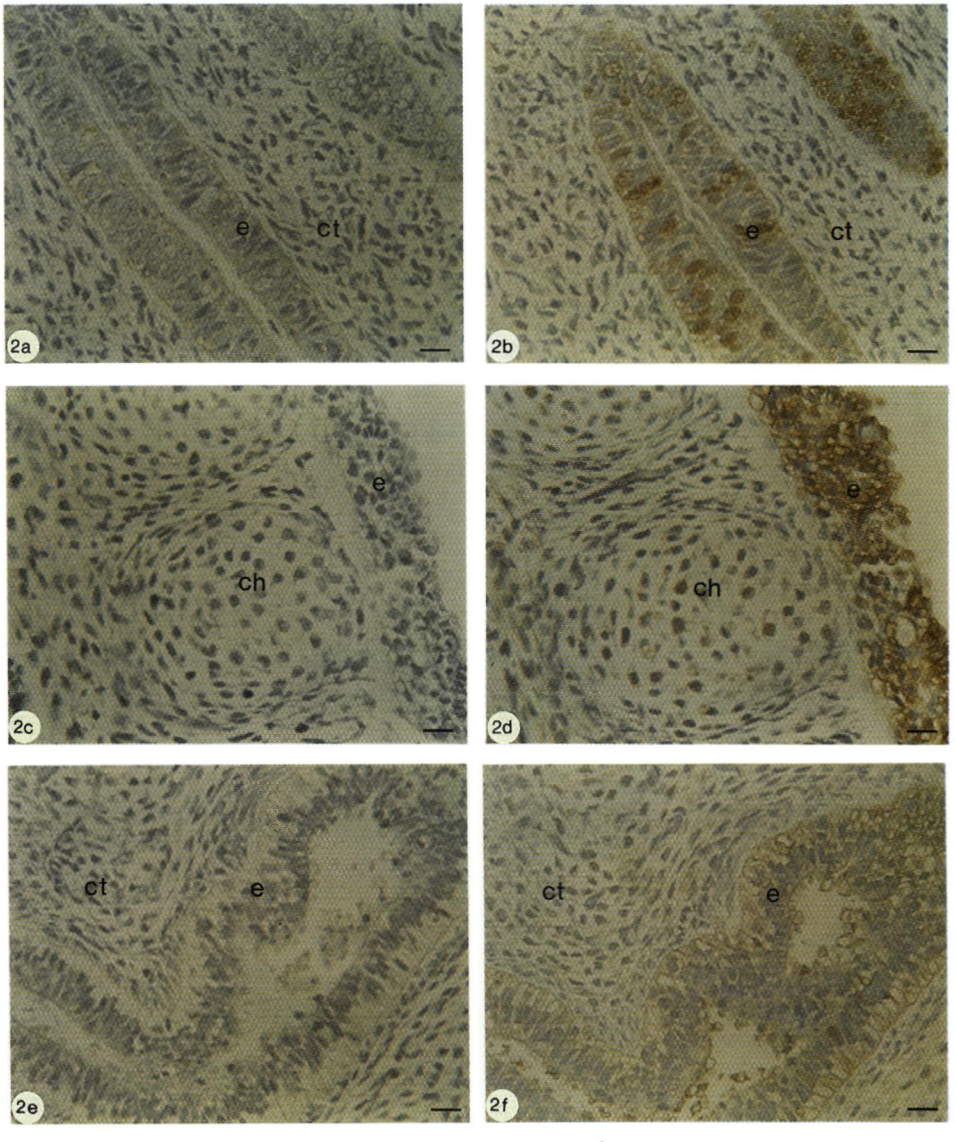

Fig. 2. *Localization of GST Alpha (a,c,e) and GST Pi (b,d,f) in primary urinary duct (a,b), trachea (c,d) and lung in glandulary phase (e,f). ch, chondrocyte; ct connective tissue; e, epithelium. Bars indicate 4 μm.*

cells in the pia mater. Ependyma cells and brain tissue showed no immunoreactivity.

Trophoblastic tissue.

In early trophoblastic tissue decidua basalis cells as well as cytotrophoblast cells stained strongly with the GST Pi antibody, whereas no immunoreactivity was seen in syncytiotrophoblast cells. Some macrophages (Hoffbauer's cells) were weakly stained for GST Pi. Staining observed in trophoblastic tissue was only cytoplasmic. Trophoblastic tissue did not contain GST Alpha immunoreactivity.

Discussion.

Since GST classes Alpha, Mu, Pi and Theta, are active towards different substrates, differential expression of these classes may be important in detoxification. The distribution of GST isoenzymes in human newborn or adult tissues has been actively studied [1, 2, 7], but little is known about fetal and embryonic tissues. This is particularly important since some drugs and chemicals may cross the placenta and accumulate in the fetus [8]. We have now shown the cellular localization of GST Alpha and Pi in embryonic tissues at 8 weeks' gestational age.

No reports on immunohistochemical staining for GST Alpha and Pi in the embryonic gastrointestinal tract have been published previously, although much is known about the localization in adults. In the adult oesophagus GST Pi expression was demonstrated in submucosal glands and in the basal layers of the squamous stratified epithelium [14]. In the adult gastric epithelium GST Alpha was expressed in oxyntic cells, whereas GST Pi staining tended to concentrate in mucous secretory cells [12, 14, 15]. In gastric glands, parietal, oxyntic and mucous neck cells weakly stained for GST Pi, whereas chief cells were negative [12, 14, 15]. In small intestinal epithelium GST Pi was seen in absorptive cells and mucous-secretory cells of both villi and crypts of Lieberkühn, whereas GST Alpha was only found in the tips of the villi [14, 15, 16]. No GST Pi was found in the duodenal submucosal glands of Brünner [14]. In colon GST Pi is the major isoenzyme, being mainly expressed in crypt cells [16 - 21]. The localization of GST Alpha and Pi in the embryonic gastrointestinal tract is different from adults, since we demonstrated GST Alpha and Pi throughout the epithelium of the embryonic gastrointestinal tract. The isoenzymes probably are down-regulated in some types of cells later on in gestation or after birth. At 8 weeks' gestational age it is not yet possible to discriminate between different cell types in the gastrointestinal epithelium. Expression of GST Alpha and Pi along the digestive tract of

embryo, fetus and adult may reflect the role of these proteins in the elimination of toxic and carcinogenic compounds

The heterogenous staining of GST Alpha and Pi in embryonic hepatocytes may reflect differences in developmental stage Hiley *et al* [9] found GST Alpha in some hepatocytes and GST Pi in most hepatocytes and in biliary duct epithelium at 12 weeks' gestational age The haematopoietic cells were not stained They suggested that during gestation the GST Pi locus will be down-regulated in hepatocytes but not in epithelium [9] At 8 ratios gestational age no biliary ducts are present in the liver In adult liver localization patterns are different, hepatocytes only express GST Alpha, whereas biliary duct epithelium only contains GST Pi [14 - 16] The function of nuclear staining of GST, as found by us and others [14, 16] remains to be clarified Nuclear staining with GST Pi has previously been reported in healthy and diseased uterine cervix cells [22] In rats it has been postulated that class Mu GST subunits are involved in nuclear RNA processing [23] Any relationship between nuclear staining and its function remains to be clarified

No reports on the expression of GST Alpha or Pi in embryonic pancreatic tissue have previously been published The staining pattern of GST Pi antibodies correlates with staining found in adult pancreas, sharply outlining the centroacinar and ductular structures, and leaving the acini and islets unstained Later on in gestation GST Alpha may be up-regulated, since Campbell *et al* [15] have shown that in adult pancreas GST Alpha antibodies stained the cells lining ducts and acini, particularly the nuclei of the acinar cells

The absence of GST Alpha in embryonic renal tissue has previously been shown by Faulder *et al* [24], who demonstrated that GST Alpha was not detectable in cytosol from kidney until 10 weeks' post natal age On the other hand, Strange *et al* [10] found that before 20 weeks' gestational age the collecting tubes and primitive Bowman's capsules express GST Alpha, whereas primitive glomeruli and mesenchymal tissue do not Between 20 and 35 ratios gestational age, as the primitive nephron elongates and fuses with the collecting tube, its entire length showed GST Alpha and Pi immunoreactivity [10] Generally speaking, the expression of GST Alpha and Pi was similar in fetuses before 35 weeks of gestation [10] Both GST Alpha and Pi were expressed along the entire developing nephron until about 35 weeks, after which period expression of GST Alpha was restricted to the proximal tubule, and of GST Pi to the collection tubules and distal part of the loop of Henle [10] Neither locus was expressed in the glomerulus This expression pattern is found in adults as well [14, 15] The physiological significance of changes in expression of GST Alpha and Pi in the developing nephron is unclear The proximal tubule is the site of considerable metabolic activity and oxygen consumption and presumably the production of potentially harmful oxygen-derived free radicals and hydroperoxides GST

Alpha, but not Pi isoenzymes, catalyze the detoxification of organic peroxides and may be important in the protection of cell structures The presence of GST Pi in nephric structures such as loop of Henle, distal convoluted tubule, collecting duct, and further along the urinary tract in the ureter and urinary bladder [10], suggests a possible role for GST Pi, and to a lesser extent for GST Alpha in the transport or elimination of toxic substances in the urine This may give significant protection against toxic compounds after birth, but may also be involved in the detoxification of compounds in urine excreted by the embryo and fetus from as early as 11 weeks [25]

In contrast to our results, GST Alpha has previously been shown in cytosol from embryonic adrenal gland at 10 weeks' gestational age [24] In adult adrenal gland Campbell *et al* [15] have demonstrated high GST Alpha immunoreactivity in deep reticular layers, whereas GST Pi showed weaker and more diffuse staining in parenchyma throughout all the layers of the gland The difference in presence of GST Alpha and Pi in adult as compared to fetal adrenal glands suggests that GST Alpha will be up-regulated in the parenchyma during gestation, whereas GST Pi will be down-regulated

We found no GST Alpha immunoreactivity in embryonic trachea, but GST Pi was present in epithelium and some chondrocytes Terrier *et al* [14] demonstrated GST Pi in adult tracheal chondrocytes, but in contrast to our results not in the cartilaginous matrix This may suggest that GST Pi will be down-regulated during gestation or after birth

We were not able to demonstrate GST Alpha immunoreactivity in embryonic lung, although GST Alpha expression in first half of gestation has been shown by others [10, 26] Strange *et al* [10] found GST Alpha in both major and distal airway cells throughout development Biochemical studies showed apparent down-regulation of GST Pi during the first half of gestation, in contrast to a continuous but weak expression of GST Alpha [26] The period of weeks 16 24 of gestation is crucial for development of the lung, since it changes from a glandular-like secretory organ to an organ capable of gaseous exchange Early in gestation GST Pi is present in epithelial cells but as development proceeds expression is maintained in proximal but not in more distal airway cells [10] This down regulation of GST Pi during the first half of gestation was also demonstrated by biochemical studies [26] and appears to precede phenotypic differentiation of the distal airways [10] The expression of GST Pi in an early stage of development suggests an additional role of this isoenzyme other than the protection of the respiratory tract against air pollutants

Carder *et al* [27] studied 21 fetal brains of 12 to 22 weeks' gestational age but in contrast to our results did not detect GST Alpha This discrepancy may be due to the different antibodies used Our antibody cross-reacts with all known GST Alpha hetero- and

homodimers; GST A1-1, GST A1 2 and GST A2-2 [13] Carder *et al* [27] showed that GST Pi was expressed from as early as 12 weeks' gestational age In the adult brain GST Pi was shown in choroid plexus, vascular endothelium, ventricular lining cells, pia-arachnoid and astrocytes, whereas neurons were consistently negative In general, GST Pi is localized at the site of the blood-cerebrospinal fluid (CSF), blood brain, CSF-brain and pia-arachnoid barriers, where it may regulate neuronal exposure to potentially toxic substances derived from blood or CSF Expression of GST Alpha and Pi this early in gestation may be significant in the protection of the developing human brain

No immunohistochemical data on the expression of GST Alpha or GST Pi in embryonic trophoblastic tissue were available until now Pacifici *et al* [8] were able to measure GST activity in placental tissue homogenates from as early as 11 weeks' gestational age No relationship between GST activity and gestational age (11-25 weeks) was found, demonstrating that placental GST develops before the 11th week of gestation and does not undergo rapid changes during the second trimester [8]

In summary, our results demonstrate that both both GST Alpha and Pi immunoreactive protein are present early in human embryonic life at 8 weeks' gestational age Since we only stained for immunoreactive protein and did not measure enzyme activity we cannot exclude that premature forms of GST are present at this stage of human embryonic life Since some drugs and chemicals may cross the placenta and accumulate in fetal tissues, a wide distribution of GST early in development may be important The differences between our results in embryonic tissues and those obtained by other investigators may be due to differences in gestational age, the specific characteristics of the antibodies used, whereas interindividual differences also cannot be ruled out The early expression of GST Alpha and GST Pi in the epithelia of the urinary and digestive tracts and the respiratory system, which are three major systems involved in elimination and transport of toxic substances in the adult human body, supports the importance of these GST in detoxification mechanisms in human life

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CHAPTER 5

Glutathione and glutathione-related enzymes in hypertensive disorders of pregnancy

PARAGRAPH 5.1

Glutathione and glutathione-related enzymes in decidua and placenta of women with preeclampsia

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ABSTRACT.

Objective: To investigate a possible pathophysiological involvement of glutathione and glutathione-related enzymes, being major detoxicating and oxygen free radical scavenging systems, in placental and decidual tissue in preeclampsia

Design: A prospective study

Participants: Seventeen normotensive pregnant women and 24 preeclamptic women of whom 10 showed the HELLP syndrome

Methods: Glutathione (GSH) levels, glutathione S-transferase activity (GST), total (TGPX, being non-selenium dependent) and selenium dependent glutathione peroxidase activity (SeGPX) were assessed in placental and decidual tissues, collected at caesarean section.

Main outcome measures: Decidual levels were compared to placental levels and levels in preeclampsia were compared to those in normotensive pregnancy by the Mann-Whitney U test Clinical data were correlated with GSH, GST, TGPX and SeGPX levels by Spearman's correlation test.

Results: GSH levels, TGPX and SeGPX activities were significantly higher in decidual tissue as compared to placental tissue ($P < 0.0001$). GSH levels were elevated in preeclampsia as compared to normotensive pregnancy for both decidual ($P < 0.01$) and placental ($P < 0.05$) tissues GST activity was not significantly different between the two groups In preeclampsia TGPX activity was elevated in placental tissue ($P < 0.05$) and SeGPX was higher in decidual tissue ($P < 0.001$) GSH levels correlated significantly with diastolic blood pressure and serum urate levels in both decidual and placental tissue and with platelet count, the degree of proteinuria, serum creatinine, AST, ALT and LDH levels in decidual tissue only

Conclusions: Enhanced glutathione concentrations and glutathione peroxidase activities in placental and decidual tissues in preeclampsia were found, probably acting as a compensatory mechanism to prevent further generation of lipid peroxides and oxygen radicals in the placenta or the feto-maternal interface.

INTRODUCTION.

Hypertension complicates 6 20 % of all pregnancies and ranks amongst the four most common causes of maternal and perinatal mortality in the world [1] The etiology of this gestational disorder is still largely unknown Increasing evidence suggests an impairment of endothelial cell function, possibly mediated by oxygen free radicals, lipid peroxides or other toxins, as crucial determinants in the development of this gestational disorder [2, 3] An impaired placental perfusion or an increased activity of the decidual lymphoid tissue due to immunologic maladaptation, could lead to an excessive production of oxygen free radicals and/or lipid peroxides acting locally or being spilled in the maternal circulation Lipid peroxides activate cyclo oxygenase and impair endothelial prostacyclin synthetase, thus leading to decreased prostacyclin levels [4] In normal conditions a variety of antioxidant mechanisms serve to control peroxidative processes [5], but in preeclampsia, an imbalance between lipid peroxidation and antioxidant mechanisms could impair normal endothelial function [6]

Glutathione and glutathione-related enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds and reactive oxygen species Glutathione can act either as a substrate in the cytosolic GSH-redox cycle or directly inactivate reactive oxygen species, such as the oxygen radicals O_2 and OH Glutathione is present in all cells at high concentrations (0 5 - 10 mM), most of which is in the reduced form [7]

Glutathione S-transferases (GSTs) are enzymes catalyzing the nucleophilic addition of glutathione to electrophilic centres of a wide variety of compounds They can also serve as transport proteins for a broad range of lipophilic compounds, such as bilirubin, bile acids, steroid hormones and various xenobiotics [8, 9]

Glutathione peroxidases (GPX) are enzymes that catalyze the reduction of organic hydroperoxides (lipid hydroperoxides, DNA hydroperoxides) and hydrogen peroxide Two major types of GPX have been found of which one type contains selenium (SeGPX), being active with both organic hydroperoxides and hydrogen peroxide The second type of GPX (TGPX) consists of proteins that do not depend on selenium and have negligible activity with hydrogen peroxide This class comprises mainly glutathione S transferases [10]

In this study glutathione levels, glutathione S-transferase and glutathione peroxidase activities were studied in placental and decidual tissues of preeclamptic and normotensive pregnancies

Table 1. Study group characteristics.

	Normotensive pregnancy (n = 17)	*Preeclamptic pregnancy (n = 24)*
Maternal age (yr)	33 (22 - 42)	29.5 (22 - 39)
Gestational age (wk)	38^{+5} (37^{+4} - 41^{+3})	33^{+6}* (26^{+3} - 39)
Parity	1 (0 - 3)	0* (0 - 2)
Diastolic blood pressure (K4; mm Hg)	75 (58 - 86)	110* (95 - 120)
Hb (g/dL)	11.8 (10.7 - 13.4)	12.0 (10.4 - 14.4)
Ht (L/L)	0.36 (0.32 - 0.41)	0.36 (0.3 - 0.4)
Platelet count ($*10^9$/L)	217 (150 - 301)	93* (31 - 305)
Serum creatinine (μmol/L)	60 (54 - 83)	77* (52 - 108)
Serum uric acid (mmol/L)	0.28 (0.17 - 0.36)	0.39* (0.22 - 0.55)
Serum AST (U/L)	10 (5 - 14)	67* (7 - 510)
Serum ALT (U/L)	7 (4 - 11)	56* (5 - 333)
Serum LDH (U/L)	208 (182 - 302)	534* (206 - 1607)
Proteinuria (g/L)	-	3.81* (0.3 - 27.6)

Values are given as median (range).
** $P < 0.01$ normotensive pregnancy vs preeclampsia.*

MATERIALS AND METHODS.

Study populations.

Two groups of women were studied: 17 normotensive pregnant and 24 preeclamptic women, 10 of whom developed preeclampsia complicated by the HELLP (Haemolysis Elevated Liver enzymes Low Platelets) syndrome The experimental protocol was approved by the institutional review board of the University Hospital Nijmegen. Except for two women in the preeclamptic group, all women were delivered by caesarean section (SC; elective SC due to cephalo-pelvic disproportion in the control group and emergency SC in the preeclamptic group either for deteriorating maternal or fetal condition). Preeclampsia was defined as pregnancy induced hypertension (diastolic blood-pressure 90 mm Hg on two or more consecutive occasions, each more than 4 hours apart) and concordant proteinuria (urinary protein > 300 mg/L) [11]. The HELLP

syndrome was defined as Haemolysis, defined as increased lactate dehydrogenase activity (LDH > 600 U/l), Elevated Liver Enzymes, defined as increased AST (> 70 U/l) and ALT (> 70 U/l) activity and Low Platelets, defined as a platelet count < 100 x $10^3/mm^3$ [12]. Blood pressure was taken in sitting position with a sphygmomanometer Diastole was recorded at phase IV Korotkoff sound. Urinary protein was determined in 24 hours urine samples. The characteristics of the women involved in this study are depicted in Table 1.

Tissue preparation and biochemical analysis.

Fragments of placental and decidual tissue were excised during caesarean section and frozen within 20 minutes after excision Tissue fragments (20 - 100 mg) were thawed and homogenized in 10 volumes ice-cold homogenizing buffer (250 mM sucrose, 20 mM Tris-HCl, 1 mM dithriothreitol, pH 7.4) with 10 strokes in small glass-glass homogenizers. The homogenates were centrifuged at 150,000 x *g* at 4°C for 1 h. Supernatants were frozen in liquid nitrogen and stored at -20°C in small portions

Protein contents were determined using the method of Lowry *et al.* with bovine serum albumin as a standard [13].

Total GSH was determined by HPLC after labelling with monobromobimane as described previously [14].

Glutathione S-transferase activity was determined as described earlier by the method of Habig *et al.* using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate [15]

Glutathione peroxidase enzyme activity was measured with hydrogen peroxide (selenium dependent activity, SeGPX) and t-butylhydroperoxide (total activity, being non-selenium dependent, TGPX) (Sigma Chemical Company, St Louis, MO, USA) as substrates, essentially as described by Howie *et al.* [16]. GSH levels and activities of GST, TGPX and SeGPX activities were calculated per mg total cytosolic protein

Statistical evaluations.

Comparisons of differences between placental and decidual tissues on the one hand and preeclamptic women and normotensive controls on the other were performed by the Mann-Whitney U test. Correlations between the parameters were tested with the Spearman's rank correlation test Differences were considered significant if the *P* value was below 0 05.

Table 2 Glutathione levels glutathione S transferase, total and selenium dependent glutathione peroxidase activities in placental and decidual tissue of preeclamptic and normotensive pregnancies

	DECIDUA		PLACENTA	
	Normotensive pregnancy (n = 17)	*Preeclamptic pregnancy (n = 22)*	*Normotensive pregnancy (n = 17)*	*Preeclamptic pregnancy (n = 24)*
GSH (nmol/mg protein)	115 5* (13 3 - 240 0)	185 6[$]* (59 5 354 7)	25 6 (12 9 - 49 7)	38 4[¶] (9 6 111 3)
GST (nmol/min/mg protein)	217 (111 - 518)	250 (111 458)	202 (80 - 318 5)	191 (107 5 - 458)
TGPX (nmol/min/mg protein)	55 5[§] (24 5 - 101)	65 (35 - 167)	46 (30 5 - 75 5)	60[¶] (29 5 - 116 5)
SeGPX (nmol/min/mg protein)	119 (106 - 169)	139 3[§ §] (107 5- 173 5)	106 5 (78 5 171)	119 5 (85 5 170 5)

Results are presented as median (range)
[$] *P < 0 05, * P < 0 0001, decidual vs placental tissue*
[¶] *P < 0 05, [§] P < 0 01, * P < 0 001, preeclamptic vs normotensive pregnancy*

RESULTS.

Decidual GSH levels were significantly higher as compared to placental levels in both normotensive controls and preeclamptic pregnancies GSH levels were significantly higher in both decidual tissue and placental tissue in preeclampsia as compared to normotensive pregnancy (Table 2, Fig 1) Decidual and placental GSH levels correlated significantly with several haematological and biochemical parameters reflecting the severity of the disease (Table 3)

GST activities in decidual and placental tissues were not significantly different Decidual GST levels correlated significantly with several parameters reflecting the severity of the disease (Table 3)

Decidual TGPX activity was significantly higher than the corresponding placental activity in the control group Placental TGPX activity, in contrast with that of decidua, was significantly higher in the preeclamptic group as compared to the normotensive group

Decidual SeGPX activity in the preeclamptic group was significantly higher than the corresponding placental activity (Table 2, Figs 1) and decidual SeGPX activity was

Table 3. Correlations between glutathione, glutathione S-transferase, glutathione peroxidases and several parameters reflecting the severity of the disease in preeclamptic pregnancy

	Decidua		*Placenta*	
GLUTATHIONE (GSH)				
GSH vs K4	$r = 0.57$	$P = 0.0001$	$r = 0.39$	$P = 0.01$
GSH vs proteinuria	$r = 0.43$	$P = 0.047$	*N.S.*	
GSH vs platelet count	$r = -0.36$	$P = 0.03$	*N.S*	
GSH vs creatinine	$r = 0.40$	$P = 0.01$	*N.S.*	
GSH vs urate	$r = 0.42$	$P = 0.0078$	$r = 0.34$	$P = 0.03$
GSH vs AST	$r = 0.46$	$P = 0.005$	*N.S*	
GSH vs ALT	$r = 0.43$	$P = 0.0064$	*N.S*	
GSH vs LDH	$r = 0.45$	$P = 0.0069$	*N.S*	
GLUTATHIONE S-TRANSFERASE (GST)				
GST vs RR	$r = 0.41$	$P = 0.0097$	*N.S*	
GST vs Hb	$r = 0.33$	$P = 0.04$	*N.S*	
GST vs creatinine	$r = 0.36$	$P = 0.03$	*N.S*	
GLUTATHIONE PEROXIDASE (TGPX)				
TGPX vs LDH	*N.S.*		$r = 0.34$	$P = 0.04$

significantly higher in preeclamptic pregnancy as compared to normotensive pregnancy (Table 2).

TGPX activity correlated significantly with SeGPX activity in both decidual and placental tissue ($r = 0.67$, $P < 0.0001$; $r = 0.60$, $P < 0.0001$, respectively).

DISCUSSION.

An imbalance between lipid peroxides and oxygen free radicals on the one hand and detoxicating and scavenging substances on the other may contribute to the etiology or pathophysiology of preeclampsia [3, 17]. Most organisms are equipped with enzymatic and non-enzymatic defence mechanisms against oxidants and other toxic compounds. Important antioxidant and detoxicating enzymes are superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione S-transferase. Non-enzymatic

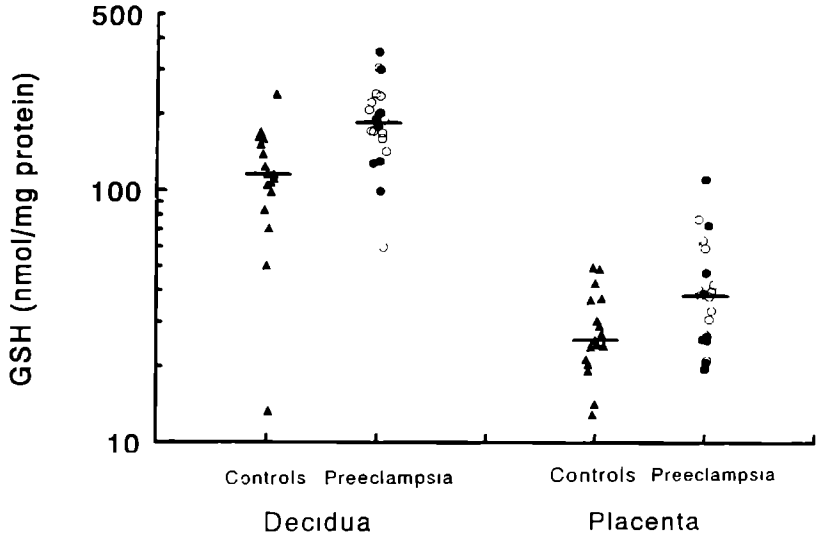

Fig 1 *Decidual and placental glutathione (GSH) concentrations in normotensive pregnancy (▲), and preeclampsia with (●) or without the HELLP syndrome (○) Bars indicate median levels*

detoxification is achieved by many different agents such as transferrin ceruloplasmin lactoferrin vitamin E, vitamin C, uric acid and thiols such as glutathione, cysteamine and cysteine For example, activities of CuZn SOD and tissue levels of vitamin E were shown to be significantly lower in placentas of preeclamptic as compared to those of normotensive pregnant women [18] Since the glutathione system in a quantitative sense is one of the most important protective systems in humans we focussed our attention on glutathione and glutathione related enzymes in both placental and decidual tissue the latter being studied for the first time in this respect

Decidual glutathione levels were much higher as compared to placental glutathione levels in both preeclampsia and normotensive pregnancy In fact, the decidual GSH levels appeared to be much higher than that in any other human tissue examined previously [14, 19, 20] This high decidual level may point to a pronounced protective role of this tissue, either protecting the mother against peroxides or oxygen free radicals produced by the feto-maternal interface or the placenta [21], or protecting the

fetus against such harmful compounds [22].

We found considerably higher levels of glutathione in placental and especially decidual tissue in preeclampsia as compared to those in normotensive pregnancy. Enhanced lipid peroxidation may be involved in the foam-cell formation of decidua and in the pathogenesis of preeclampsia [23]. GSH can inhibit lipid peroxidation via membrane bound GPX [24]. GSH levels may point to a compensating mechanism in order to prevent excessive lipid peroxidation. Human decidua mainly produces thromboxane B2 (TxB2), however, after addition of reduced glutathione prostaglandin E2 became the main product [25]. Cytosols from decidua vera and placenta were most effective in stimulating synthesis of prostaglandin E2. Reduced GSH is able to increase the biosynthesis of PGE_2 at the expense of other prostaglandins, both in the presence or absence of these cytosols [26]. Preeclampsia is associated with a change of the prostaglandin/thromboxane balance in favour of thromboxane formation. An increase in decidual and placental tissue glutathione concentration may, besides prevention of excessive lipid peroxidation point to a compensatory mechanism in order to enhance prostaglandin production. Enhanced placental GSH levels in preeclamptic women are in accordance with a previous report [27].

The decidual glutathione S-transferase (GST) activity was slightly enhanced in preeclampsia as compared to normotensive pregnancy, but the difference was not statistically significant. There is a possibility of a yet unknown toxin produced by the feto-maternal interface. This factor may be detoxified in a reaction with glutathione catalyzed by GST. Until now there's only one report investigating placental GST activity in preeclampsia and no difference between preeclamptic and normotensive pregnancy was found [28], which is in agreement with our results.

We found a significantly higher SeGPX activity in decidual tissue and significantly higher TGPX activities in placental tissue of preeclamptic pregnancy as compared to normotensive pregnancy. Interestingly, in normotensive pregnancy, TGPX activity in decidual tissue was significantly higher than in placental tissue, whereas in preeclamptic pregnancy, SeGPX activity was significantly higher in decidual tissue as compared to placental tissue. This may point to a differential upgrading of peroxidases in these tissues. In preeclampsia the relative upgrading of SeGPX is in favour of decidual tissue and the upgrading of TGPX is in favour of placental tissue. TGPX activity correlated significantly with SeGPX activity in decidual and placental tissue indicating that GPX activity in placental and decidual tissue is mainly involved in detoxifying organic hydroperoxides, and not H_2O_2. Conflicting results have been published on placental glutathione peroxidase activity in preeclampsia, varying from

decreased activities to unchanged activities [28, 29]. Glutathione peroxidase activity [30], and messenger RNA levels of glutathione peroxidase [18] were reported to be lower in placental tissue of preeclamptic pregnancies as compared to those in normotensive pregnancies whereas levels of lipid peroxides were higher [21] The discrepancy between our results and those reported in literature could be due to the fact that our patients did have a rather severe preeclampsia, leading to an even more enhanced compensatory mechanism. There seems to be an interaction between lipid peroxides, the prostaglandin/thromboxane system and glutathione peroxidases Lipid peroxides, levels of which are elevated in the maternal circulation of hypertensive pregnant women [31], may stimulate prostaglandin H2 synthase and increase thromboxane and oxygen radical production, which subsequently increases the formation of lipid peroxides [30] Cyclooxygenase activity not only generates thromboxane but also oxygen radicals [32] Glutathione peroxidases inactivate peroxides, thereby diminishing peroxide mediated stimulation of prostaglandin synthase Inhibition of glutathione peroxidase activity in normal placentas resulted in a dose dependent increase in placental production of both lipid peroxides and thromboxane, without affecting prostacyclin, so the ratio of thromboxane to prostacyclin progressively increased [30] The thromboxane/prostacyclin and lipid peroxide/prostacyclin ratios were shown to be threefold higher in preeclamptic placenta as compared to normal placenta [21] In this respect, the increased peroxidase activities we observed may act as a compensatory mechanism, both in placental and decidual tissues.

In conclusion, we found enhanced glutathione concentrations and glutathione peroxidase activities in placental and decidual tissues in preeclampsia, which significantly correlated with the severity of the disease This most probably represents a compensatory mechanism to (further) prevent generation of lipid peroxides and oxygen radicals in the feto-maternal interface, in order to prevent damage to the fetus or the mother

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PARAGRAPH 5.2

Whole blood glutathione levels and glutathione / haemoglobin ratios in pregnancies complicated by preeclampsia or the Haemolysis Elevated Liver enzymes Low Platelets syndrome

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ABSTRACT.

Objective: To investigate the pathophysiologic involvement of glutathione in pregnancies complicated by preeclampsia or the Haemolysis Elevated Liver enzymes Low Platelets syndrome (HELLP)

Methods: Total whole blood glutathione levels were measured by high performance liquid chromatography in 23 women with pregnancies complicated by preeclampsia or the HELLP syndrome, and 22 normotensive gravidas Total glutathione levels and the total glutathione / haemoglobin ratios of patients were compared to those of controls by the Mann Whitney U-test

Results: Median total glutathione levels were lower in hypertensive pregnancies than in normotensive pregnancies (647 [range 268 986] and 750 [range 495 - 1572] μmol/L, $P = 0\ 05$) The median total glutathione / haemoglobin ratios were significantly lower in hypertensive pregnancies than in normotensive pregnancies (0 079 [range 0 033 0 122] and 0.101 [range 0 073 - 0 210], $P = 0\ 02$)

Conclusion: Decreased total glutathione levels in maternal whole blood may indicate decreased detoxificating or free radical scavenging capacity in pregnancies complicated by preeclampsia or the HELLP syndrome

INTRODUCTION.

Hypertension complicates 6 - 20% of all pregnancies and ranks amongst the four most common causes of maternal as well as perinatal mortality in the world [1] The etiology of this gestational disorder is largely unknown Increasing evidence suggests an endothelial cell dysfunction, possibly mediated by oxygen free radicals, lipid peroxides and other toxins, as a crucial determinant in the development of this gestational disorder [2] Impaired placental perfusion or increased activity of the decidual lymphoid tissue due to immunologic maladaptation could lead to an excessive production of oxygen free radicals and/or lipid peroxides, either acting locally or being spilled in the maternal circulation Lipid peroxides may activate cyclo-oxygenase and impair endothelial prostacyclin synthetase, thus leading to decreased prostacyclin levels [3] Under normal conditions a variety of antioxidant mechanisms serve to control peroxidative processes [4], but in preeclampsia an imbalance between lipid peroxidation and antioxidant mechanisms could impair normal endothelial function [2, 5]

Glutathione and glutathione-related enzymes are involved in the metabolism and detoxification of carcinogenic and cytotoxic compounds, including reactive oxygen species. Glutathione can either act as a substrate in the cytosolic glutathione-redox cycle or directly inactivate reactive oxygen species, such as O_2 and OH. Glutathione is present in cells in high concentrations (0.5 - 10 mM), most of which (more than 99%) exists in the reduced form. Oxidized glutathione is either converted into the reduced form by glutathione reductase or transported out of the cell. Also chemical stress leading to extra conjugation with glutathione may result in loss of glutathione [6]. Erythrocytes and liver contain the highest GSH redox activity [7]. The amounts in plasma only represent less than 1% of the total whole blood glutathione pool, which is primarily localized in erythrocytes [8, 9].

In the present study maternal whole blood total glutathione levels were measured in pregnancies complicated by preeclampsia or the Haemolysis Elevated Liver enzymes Low Platelets syndrome (HELLP) and compared to levels in normotensive pregnancy. We also calculated the total glutathione / haemoglobin ratio to account for possible variations in haemoconcentration.

Materials and Methods.

Two groups of pregnant women were studied. 22 had normotensive, uncomplicated pregnancies, and 23 had pregnancies complicated by preeclampsia (n = 8), the HELLP syndrome (n = 6), or both (n = 9). Preeclampsia was defined as pregnancy-induced hypertension (diastolic blood pressure (BP) ≥ 90 mm Hg on two or more consecutive occasions, each more than 4 hours apart) and concordant proteinuria (urinary protein exceeding 0.30 g/L) [10]. BP was taken in sitting position with a sphygmomanometer. Diastole was recorded at phase IV Korotkoff sound. The HELLP syndrome was defined as Haemolysis, defined as increased lactate dehydrogenase activity (LDH above 600 U/L), Elevated Liver enzymes, defined as increased aspartate aminotransferase (above 70 U/L) and alanine aminotransferase (above 70 U/L) activity and Low Platelets, defined as a platelet count under 100 x 10^3/mm^3 [11].

Antecubital maternal venous blood samples were taken in sitting position after 5 minutes of rest in 4 mL ethylenediaminetetra-acetic acid tubes (Becton and Dickinson, Grenoble, France). Immediately after sampling, 1 mL of whole blood was diluted with 1 mL of a 20% (w/v) aqueous solution of trichloroacetic acid. The mixture was shaken vigorously and frozen at -20° C until use. After thawing, the mixture was centrifuged for 10 minutes at 13,000 x g, and 10 - 20 μL of the clear supernatant was used for

estimation of the glutathione content Total glutathione levels were determined by high performance liquid chromatography after labelling with monobromobimane, essentially as described previously [12].

Haemoglobin, haematocrit, platelet count, creatinine, urate, aspartate aminotransferase, alanine aminotransferase, LDH and proteinuria were analyzed routinely at the department of Clinical Chemistry of the University Hospital St. Radboud Data are expressed as medians (range) Groups were compared by the Mann-Whitney U-test, and considered significant at $P < 0\ 05$

Table 1 Population characteristics

	Hypertensive pregnancy (n = 23)	*Normotensive pregnancy (n = 22)*
Maternal age (yrs)	28 (22 - 36)	33 5 (26 - 40)*
Gestational age (wks)	32 [2] (18^{+3} - 39^{+6})	36^{+5} (35^{+4} - 37 [5])[1]
Parity	0 (0 - 2)	1 (0 - 2)[1]
Diastolic BP (K 4, mm Hg)	105 (39 - 120)	73 5 (65 - 85)[5]
Hb (mmol/L)	7 9 (5 5 - 9 5)	7 45 (6 - 8 1)
Ht (L/L)	0 37 (0 26 - 0 45)	0 36 (0 30 - 0 40)
Platelet count ($*10^9$/L)	88 (26 - 303)	
Serum creatinine (μmol/L)	82 (52 166)	
Serum uric acid (mmol/L)	0 42 (0 26 0 59)	
Serum aspartate aminotransferase (U/L)	125 (11 - 1350)	
Serum alanine aminotransferase (U/L)	105 (6 - 1140)	
Serum LDH (U/L)	807 (207 - 3210)	
Proteinuria (g/L)	1 0 (0 03 - 27 6)	-

Results are presented as median (range)
** $P = 0\ 0001$, [1] $P = 0\ 0007$, [5] $P < 0\ 0001$ hypertensive pregnancy vs normotensive pregnancy. Mann Whitney U-test, BP = blood pressure, LDH = lactate dehydrogenase*

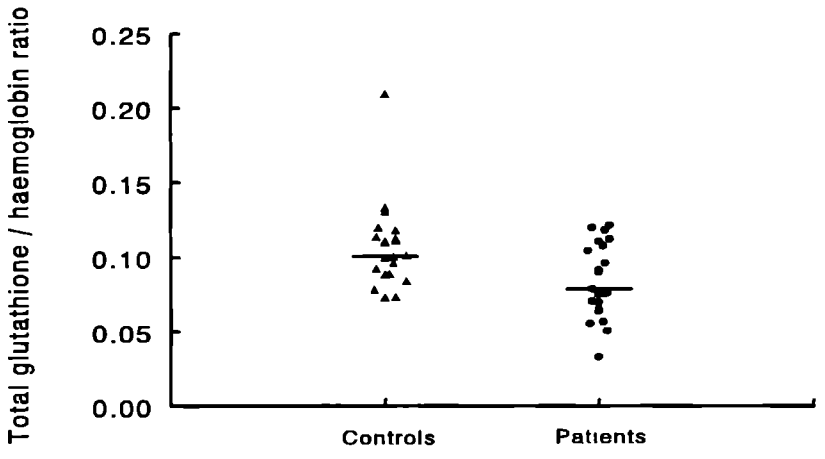

Fig. 1. *Maternal whole blood total glutathione / haemoglobin ratios in normotensive pregnancy (▲), and pregnancies complicated by preeclampia or the HELLP syndrome (●). Horizontal lines indicate mean levels.*

RESULTS.

Maternal age, parity and gestational age were significantly lower and diastolic BP was significantly higher in pregnancies complicated by preeclampsia or the HELLP syndrome as compared to normotensive, uncomplicated pregnancies (Table 1)

Median whole blood glutathione levels were lower in women with hypertension than in normotensive gravidas, reaching borderline statistical significance (647 [range: 268 - 986] and 750 [range· 495 - 1572] μmol/L, $P = 0.05$) The median glutathione / haemoglobin ratio was significantly lower in hypertensive as compared to normotensive pregnancies (median 0.079 [range: 0.033 - 0.121] and 0.101 [range. 0 073 - 0.210], $P = 0$ 02, Fig. 1).

DISCUSSION.

Median whole blood total glutathione ratios were decreased in pregnancies complicated by preeclampsia or the HELLP syndrome. Since whole blood glutathione levels mainly originate from erythrocytes [8, 9], we also calculated the total glutathione /

haemoglobin ratio to account for possible variations in haemoconcentration Whole blood total glutathione / haemoglobin ratios were significantly lower in hypertensive pregnancies as compared to those in normotensive pregnancy, suggesting a decreased detoxificating or free radical scavenging capacity

Several studies show evidence of the involvement of oxygen free radicals and lipid peroxidation products in preeclampsia and pregnancy induced hypertension [13 14] and numerous studies have been published on the antioxidant and detoxification capacity of maternal blood in hypertensive disorders of pregnancy as reviewed by Roberts *et al* [2], and Hubel *et al* [5] In this respect, several studies have been published on glutathione and glutathione-related enzymes Erythrocyte thiols, mainly consisting of glutathione [15], and glutathione levels were found to be lower in preeclampsia [16] and pregnancy-induced hypertension [17] as compared to normotensive pregnancy In addition plasma thiols, and glutathione were significantly decreased in pregnancy-induced hypertension [16, 18] In agreement with these results, significantly higher levels of non selenium dependent glutathione peroxidase activity one of the enzymes that consumes glutathione as a co factor, were found in erythrocytes and plasma in severe preeclampsia as compared to normotensive pregnancy [14] The results of our study, and those reported in the literature, suggest a compensatory increase in the glutathione peroxidase activity in maternal blood resulting in a loss of glutathione from whole blood

In our study a significantly lower gestational age was found in patients as compared to controls Non-selenium dependent peroxidase activity and lipid peroxidation products increase with advancing gestation in normotensive uncomplicated pregnancy [19], suggesting an increased peroxide or oxygen free radical load, and as a consequence, glutathione levels may decrease with advancing gestation However, no data in this respect are available.

Whole blood total glutathione / haemoglobin ratios decreased in women with advancing age [8] Since in our study maternal age was higher in controls as compared to patients, the difference in total glutathione / haemoglobin ratios would even be higher if maternal ages of both groups were completely matched

Kidney and especially liver play a pivotal role in the homeostasis of whole blood glutathione, especially in providing precursors such as cysteine to erythrocytes [9] Erythrocytes most likely play a significant role in the interorgan transport of glutathione [9] Dependence of erythrocytic glutathione concentrations on the hepatic, and to a lesser extent renal output of amino acid precursors may explain why red blood cell glutathione concentrations are often decreased in hepatic [20] or renal failure [21]

Since hepatocellular and renal impairment are hallmarks of the HELLP syndrome and preeclampsia respectively, an inadequate provision of glutathione precursors to erythrocytes, apart from a possible overconsumption due to oxidative or chemical stress may be inferred

Normalization of glutathione levels in patients with preeclampsia or the HELLP syndrome may be a promising therapy for the future In endothelial cell culture systems it was demonstrated that an enhancement of intracellular glutathione [22, 23] provided a better protection against damage by hydrogen peroxide This may be relevant for hypertensive disorders of pregnancy, which are considered endothelial disorders [2] Infusion of S-nitrosoglutathione in patients with severe preeclampsia, lowered maternal mean arterial pressure, platelet activation, and uterine artery resistance without further compromising fetal Doppler indices [24] In addition it is suggested to exert beneficial effects in the treatment of the HELLP syndrome [25] Administration of N acetylcysteine, a precursor of glutathione, may also be beneficial in the treatment of preeclampsia, because it reduces in vitro feto-placental resistance [26], and although it is proved to cross the feto-placental barrier [27], it does not seem to induce adverse effects in the fetus [28]. N acetylcysteine is easily transported into the cell where it is deacetylated, and partly used for the synthesis of reduced glutathione It is noteworthy also to be beneficial in preventing free radical oxidative damage in an animal model of myocardial damage [29]

In conclusion, low total glutathione levels have been demonstrated in maternal whole blood of patients with preeclampsia or the HELLP syndrome as compared to normotensive pregnancy This may result in decreased detoxificating or free radical scavenging capacity Administration of glutathione-enhancing agents, such as N-acetylcysteine may be beneficial in the treatment of these gestational disorders

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PARAGRAPH **5.3**

Plasma glutathione S-transferase Alpha 1-1: a more sensitive marker for hepatocellular damage than serum alanine aminotransferase in hypertensive disorders of pregnancy

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ABSTRACT.

Objective: Our purpose was to investigate the value of plasma glutathione S-transferase Alpha 1-1 (GSTA1-1) measurements in the assessment of hepatocellular damage in hypertensive disorders of pregnancy.

Study design: Patients were recruited at the Department of Obstetrics and Gynaecology of the University Hospital St. Radboud, Nijmegen, The Netherlands. Five groups of patients were studied. normotensive pregnancy (n = 87), pregnancy-induced hypertension (n = 48), preeclampsia (n = 79), the Haemolysis Elevated Liver enzymes Low Platelets syndrome (HELLP) (n = 39), and longitudinally studied normotensive pregnancy (n = 21). Blood was collected for the assessment of plasma GSTA1-1 levels and serum alanine aminotransferase (ALT) activity. Levels in hypertensive pregnancies were compared with levels in normotensive pregnancy by the Mann-Whitney U test. Patients were categorized according as to whether their levels are below (normal) or above (elevated) the upper normal reference level. The difference in relative magnitude of elevation between the two factors was determined by the Wilcoxon matched-pairs signed-rank test

Results: Plasma levels in the longitudinally studied normotensive pregnancy group did not differ between gestational ages, and were not significantly different from those of the normotensive control group. Median levels of GSTA1-1 and ALT were significantly increased ($P < 0.01$, $P < 0.0001$ respectively) in all subgroups of hypertensive pregnancies compared with those in normotensive pregnancy. When both levels were elevated, the relative magnitude of the increase of GSTA1-1 levels was significantly higher than that of ALT activity in both preeclampsia ($P < 0.01$) and the HELLP syndrome ($P < 0.0001$). Almost half of the patients with preeclampsia showed elevated levels of plasma ALT and/or GSTA1-1 levels.

Conclusion: Plasma GSTA1-1 measurements may provide a more sensitive indicator of acute hepatocellular damage in preeclampsia and the HELLP syndrome, compared with the assessment of aminotransferase activity, and therefore may allow earlier recognition of these syndromes. The clinical benefits of plasma GSTA1-1 measurements for monitoring the hepatocellular condition in the management of these patients need to be elucidated in further studies.

INTRODUCTION.

Pregnancy-induced hypertension, preeclampsia and the syndrome of haemolysis, elevated liver enzymes, and low platelets (HELLP) are major causes of maternal and perinatal morbidity and mortality Remarkably few authors have stressed the importance of the hepatic involvement in these multi-system disorders, although liver dysfunction is a frequent and important complication of hypertensive disorders in pregnancy, associated with the severity of the hypertension and the involvement of other organ systems [1] Deterioration of hepatic function is a crucial determinant in the clinical management of these pregnancies. Therefore a correct assessment of possible hepatocellular damage is essential However, serum aspartate aminotransferase (AST, EC 2 6 1 1) and alanine aminotransferase (ALT, EC 2 6 1 2) activities, the standard liver function tests, do not accurately reflect the severity of the underlying histopathologic condition in these syndromes [2, 3]. AST is found in high concentrations in heart and skeletal muscle, kidney, pancreas, and erythrocytes as well Haemolysis may contribute significantly to a rise in AST levels in addition to hepatocellular damage [4] Elevated serum ALT levels are considered to be more specific for hepatocellular damage than serum AST levels are because the concentration of ALT in liver is relatively high A sustained elevation of serum ALT levels in patients with the HELLP syndrome has been found, indicating persistent hepatocellular damage [5].

The glutathione S-transferases (GST, EC 2 5 1.18) are a multigene family of enzymes that possess many biologic functions. They catalyze the addition of glutathione to a wide variety of compounds such as xenobiotics and serve as transport proteins for lipophilic compounds [6, 7] Cytosolic GSTs are dimeric proteins, divided into four classes: Alpha, Mu, P_i, and Theta [8] Two class Alpha subunits have been identified, and two homodimeric (GSTA1-1 and GSTA2-2) and a heterodimeric enzyme (GSTA1-2) have been purified from human liver [9]

More than 2% of the soluble protein in liver consists of GST Alpha [10]. The enzyme is released in considerable quantity into the bloodstream during hepatocellular damage [7] The half life in plasma is approximately 1 hour and plasma levels will follow changes in hepatocellular damage more rapidly than will AST (half-life 17 hours) or ALT (half-life 47 hours) [11] In the current study we investigated the possible advantage of plasma GSTA1-1 levels measurements compared with that of serum ALT activity in the assessment of hepatocellular damage in pregnancy induced hypertension, preeclampsia and the HELLP syndrome

MATERIALS AND METHODS.

Study populations.

The population characteristics of the subgroups of patients studied are presented in Table 1. Some data of 21 preeclamptic patients have contributed to a preliminary study [12]. Women entered the study after informed consent was obtained. The experimental protocol was approved by the Medical Ethical Review Committee of the University Hospital St. Radboud. Pregnancy-induced hypertension (n = 48) was defined as a diastolic blood pressure ≥ 90 mm Hg on two or more consecutive occasions, each more than 4 hours apart. Preeclampsia (n = 79) was defined as pregnancy-induced hypertension and concordant proteinuria (urinary protein/creatinine ratio ≥0.30 g/10 mmol). The HELLP syndrome (n = 39; 8 were postpartum) was defined as Haemolysis, defined as increased lactate dehydrogenase (LDH) activity (>600 U/l), Elevated Liver enzymes, defined as increased AST (>70 U/l) and ALT (>70 U/l) activity and Low Platelets, defined as a platelet count $<100 \times 10^3/mm^3$ [13, 14]. Blood pressure was taken with a sphygmomanometer with the patient in the sitting position. Diastole was recorded at the phase IV Korotkoff sound. Eighty-seven women were studied during uncomplicated, normotensive pregnancies. In addition, plasma levels of GSTA1-1 were measured in 21 normotensive nulliparous pregnant women studied longitudinally for other purposes [15], who were delivered of term, healthy, appropriate-for-gestational-age infants at gestational weeks 12, 16, 20, 24, 28, 32, 36 and 38.

Table 1. Population characteristics.

	Normotensive pregnancy	*Pregnancy-induced hypertension*	*Preeclampsia*	*HELLP syndrome*
Patients (n)	87	48	79	39
Age (yr)	31 (18-39)	31 (21-40)	30 (19-40)	30 (21-39)
Gestational age (wk)	32 (20-43)	37 (26-40)	33 (18-42)	30 (25-39)

Results are presented as median; range is given in parentheses.

Analytical procedures.

Antecubital venous blood samples were taken with the patient in the sitting position after 5 minutes of rest. Blood for measurement of GSTA1-1 was drawn in 4 mL tubes, containing ethylenediaminetetraacetic acid (No. 606601 Becton-Dickinson, Grenoble, France). For the measurement of AST, ALT and LDH activity, blood was collected in 4 mL Corvac Vacutainer glass tubes (Monoject, No. 37, Sherwood Medical, Ballymoney, United Kingdom). Blood was centrifuged within 15 minutes at 3,000 x *g* for 10 minutes and plasma or serum collected. Measurements of serum AST, ALT and LDH activities were performed at the local department of clinical chemistry on a Hitachi 747 analyser (Osaka, Japan). Plasma was stored at -20°C until assessment of glutathione S-transferase Alpha 1-1 levels.

Plasma GSTA1-1 concentrations were measured with use of a recently developed enzyme linked immunosorbent assay [16]. Microtiter plates were coated overnight with purified anti-GSTA1-1 monoclonal antibody (5B11).

One hundred microliters of standard (0.04 to 20 μg/L GSTA1-1), or 1:1, 1:100 and 1·1000 diluted plasma samples were added to the wells and incubated overnight. The next day the plates were washed and incubated with rabbit anti-GST-Alpha antiserum, washed again, incubated with horseradish peroxidase-labelled swine anti-rabbit antiserum. After a final wash, plates were stained with o-phenylenediamine-hydrogen peroxide Standards and samples were measured in duplicate. A four-parameter weighted logistic regression model was used to calculate standard curves and unknowns. The assay has a detection limit of 0.04 μg/L GSTA1-1, and the intra-assay and inter-assay coefficients of variation are 2.5% and 7.3%, respectively.

Statistical analysis.

Samples were categorized according to their GSTA1-1 and ALT levels, below (normal) or above (elevated) the upper normal reference level (2 SDs above the mean). An upper normal reference level of 5.9 μg/L GSTA1-1 was calculated from plasma samples of 350 healthy non-pregnant controls [16]. Upper normal reference levels of 25 U/L and 30 U/L for AST and ALT, respectively, were used as specified by the local department of clinical chemistry.

The statistical significance of differences between median levels of GSTA1-1 and ALT between patients and healthy pregnant controls was evaluated with use of the Mann-Whitney *U* test. To avoid spurious differences, data were initially subjected to a

Kruskal-Wallis analysis. The correlations between GSTA1-1 and ALT levels were evaluated with use of Spearman's rank correlation procedure. Differences between the relative magnitudes of elevations of plasma GSTA1-1 and serum ALT levels in a subgroup of patients with preeclampsia or the HELLP syndrome who had elevated levels of GSTA1-1 and ALT were evaluated with use of the Wilcoxon Matched-pairs signed-rank test. Differences between gestational ages in the longitudinally studied group and the normotensive pregnancies were evaluated by Kruskal-Wallis analysis. All statistical analyses were performed with use of SPSS/PC+$V_{5.0}$ (SPSS, Chicago) statistical computer software.

Table 2. Longitudinal plasma GSTA1-1 concentrations in uncomplicated nulliparous pregnancy (n = 21).

	week 12	*week 16*	*week 20*	*week 24*	*week 28*	*week 32*	*week 36*	*week 38*
GSTA1-1	1.1	1.0	1.1	1.4	1.0	1.1	1.2	1.2
(μg/L)	(0.1-4.5)	(0.3-2.5)	(0.3-2.6)	(0.3-6.0)	(0.4-5.5)	(0.4-3.6)	(0.3-7.1)	(0.3-6.0)

Results are presented as median; range is given in parentheses.

Table 3. Diastolic blood pressure, plasma GSTA1-1 concentrations and serum ALT activities

	Normotensive pregnancy	*Pregnancy-induced hypertension*	*Preeclampsia*	*HELLP*
Diastolic blood pressure	70	95	100	100
(mm Hg)	(50 - 89)	(90 - 130)	(90 - 130)	(75 - 140)
GSTA1-1	1.0	1.5*	4.1§	131.7§
(μg/L)	(0.2 - 10.9)	(0.5 - 285.5)	(0.9 - 688.0)	(9.4 - 2399.0)
ALT	4	7§	15§	191§
(U/l)	(1 - 22)	(2 - 230)	(1 - 537)	(74 - 1140)

Results are presented as median; range is given in parentheses.
* $P < 0.01$, § $P < 0.0001$, *significantly different from values during normotensive pregnancy.*

Table 4 Number of women with normal or elevated GSTA1-1 concentration and/or ALT activity

	Normotensive Pregnancy	*Pregnancy-induced hypertension*	*Preeclampsia*	*HELLP*
GSTA1-1 and ALT normal	83 (95%)	41 (86%)	43 (54%)	0 (0%)
GSTA1-1 elevated , ALT normal	4 (5%)	2 (4%)	15 (19%)	0 (0%)
ALT elevated, GSTA1-1 normal	0 (0%)	1 (2%)	4 (5%)	0 (0%)
GSTA1-1 and ALT elevated	0 (0%)	4 (8%)	17 (22%)	39 (100%)

Percentage is given in parentheses

Table 5. Relative magnitude of elevation of GSTA1-1 concentration and ALT activity

	Preeclampsia	*HELLP*
GSTA1-1/UNRL	6 4*(1 3 - 116 8)	22 4§ (1 6 - 4057 3)
ALT/UNRL	2 4 (1 1 - 17 9)	6 4 (2 5 - 38 0)

Results are presented as median, range is presented in parentheses.
** $P < 0.01$, statistical significance of the difference between the ratio's of GSTA1-1 and ALT to the appropriate UNRL.*
§ $P < 0.0001$, statistical significance of the difference between the ratio's of GSTA1-1 and ALT to the appropriate UNRL

RESULTS.

There was no statistical difference between plasma GSTA1-1 levels at different gestational ages in the longitudinally studied group (Table 2), nor did these values differ from those in the normotensive pregnancy group ($P = 0.995$). In the group of 275 women studied cross-sectionally, plasma GSTA1-1 concentrations were significantly correlated with serum ALT activities ($r = 0.75$, $P < 0.00001$) Median GSTA1-1 and ALT levels were significantly increased in pregnancy-induced hypertension ($P < 0.01$, $P < 0.0001$ respectively), preeclampsia ($P < 0.0001$), and the HELLP syndrome ($P < 0.0001$) compared with normotensive pregnancy (Table 3) In patients with pregnancy-induced hypertension and preeclampsia, 14% and 46%, respectively, of these groups of patients studied showed elevated GSTA1-1 and/or ALT levels (Table 4, Fig. 1) Relative magnitudes of elevation of plasma GSTA1-1 concentrations were significantly more prominent than those of serum ALT activities in patients with preeclampsia

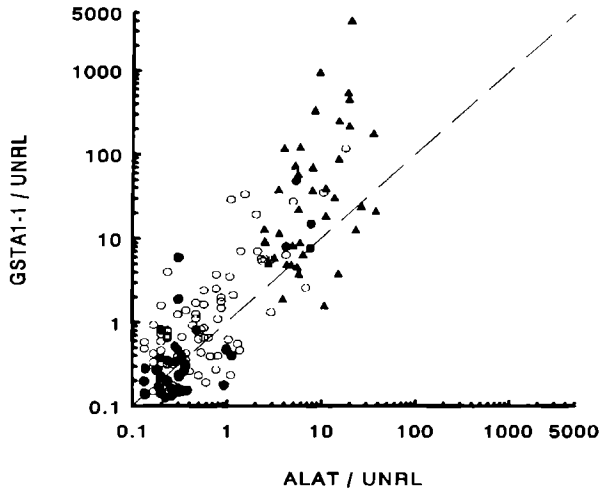

Fig. 1. *GSTA1-1 and alanine aminotransferase (ALT) as multiple of the upper normal reference level for pregnancy-induced hypertension (●), preeclampsia (○) and the HELLP syndrome (▲).*

($P < 0.01$) or the HELLP syndrome ($P < 0.0001$) who showed both elevated ALT and elevated GSTA1-1 levels (Table 5). GSTA1-1 and ALT levels are presented in Fig. 1 as multiple of the upper normal reference level.

Discussion.

This study shows significant increases of median GSTA1-1 concentrations and ALT activity in hypertensive pregnancies compared with normotensive pregnancies. About half of the patients with preeclampsia had elevated plasma GSTA1-1 and/or ALT levels, as defined by levels above the upper normal reference level. By measuring plasma GSTA1-1 levels the incidence of some degree of hepatocellular damage seems to be higher than the previously reported 24% [17, 18]. Gestation by itself does not seem to influence hepatocellular integrity because plasma levels of GSTA1-1 studied longitudinally in pregnancy did not differ among different gestational ages. The cause of impaired hepatocellular integrity in hypertensive disorders of pregnancy and especially the HELLP syndrome remains unknown. Enhanced hepatic artery resistance

may point to vasoconstriction and hypoperfusion of the hepatic vascular bed, resulting in hypoxic damage [19]. Microvascular thrombosis in small hepatic vessels could further contribute to hepatic tissue necrosis and thereby to elevated concentrations of liver enzymes in blood [14]. Plasma or serum concentrations of biochemical markers of hepatocellular damage such as AST and ALT, commonly used in the assessment of hepatocellular integrity in preeclampsia and the HELLP syndrome may be misleading if used alone. Haemolysis, a prominent feature of the HELLP syndrome, probably contributes substantially to the elevated levels of AST and LDH whereas enhanced ALT levels may in part reflect accompanying muscle injury [4, 5].

There are several reasons why measurement of GSTA1-1 could offer potential advantages over the transaminases in the investigation of hepatocellular damage in preeclampsia and the HELLP syndrome. GSTA1-1 has a molecular mass of 52 kD and is readily and rapidly released into the blood after hepatocellular damage [7]. In addition, relatively large amounts of GSTA1-1 enter the circulation because the enzyme constitutes as much as 2% of the cytosolic protein in the hepatocyte [10, 20]. In contrast to the periportal location of aminotransferases, GSTA1-1 is primarily located in centrilobular hepatocytes [21]. This location may be more relevant to the study of a syndrome characterized by enhanced hepatic artery resistance [19]

Plasma concentrations of GSTA1-1 appear to be a more sensitive indicator of acute hepatocellular damage than ALT in patients with the HELLP syndrome. Our study shows that the relative magnitude of the abnormality of plasma GSTA1-1 levels in preeclampsia and the HELLP syndrome was up to 100 times greater than that of serum ALT activity Furthermore, the elevation in GSTA1-1 levels has been previously shown to precede that of ALT by several hours in two patients with a severe HELLP syndrome [12]

Our results may point to hepatic hypoxia causing centrilobular necrosis and subsequent release of relatively large amounts of GSTA1-1 into the blood. In patients with paracetamol poisoning, halothane hepatitis, or rejection following liver transplantation, plasma GST Alpha concentrations are known to provide a more sensitive marker for acute hepatocellular damage than aminotransferase activity [22 - 24] Furthermore, when the active phase of hepatocellular damage in these conditions is over, plasma concentrations of GST Alpha rapidly revert to normal, a feature of the short plasma half-life (<90 minutes [22]), whereas aminotransferase activity may be abnormal for much longer Patients with the HELLP syndrome showed a similar quick decline of GSTA1-1 concentrations when their first attack of epigastric pain subsided and again when their pregnancies were terminated by caesarean section after their

second episode of upper abdominal pain [12] The short plasma half-life may also explain why in some of the patients the elevation of ALT was more pronounced than that of GSTA1-1. Because of the slow plasma clearance, elevated levels of ALT may still be detected several days after an acute attack of liver damage, whereas GSTA1-1 values have already normalized [12]

In conclusion, plasma GSTA1-1 measurement may provide a sensitive indicator of acute hepatocellular damage in preeclampsia and the HELLP syndrome and could have some advantage over conventional parameters of hepatocellular damage, especially in early recognition of disease The clinical benefits of plasma GSTA1-1 measurements for monitoring the hepatic condition in the management of these patients need to be elucidated in further studies

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PARAGRAPH **5.4**

Marker for liver damage in neonates born to mothers with HELLP syndrome

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The syndrome of haemolysis, elevated liver enzymes and low platelets (HELLP) is a major cause of maternal and perinatal morbidity and mortality [1]. The etiology and the characteristic pattern of maternal hepatic damage in this condition are not known. Deterioration of the mother's condition is a major determinant of the management of these pregnancies, and often results in assisted delivery of a premature infant. Little is known about the consequences of this disorder in the neonate. Thrombocytopenia has not been shown in neonates born from hypertensive pregnancies with associated maternal thrombocytopenia [2]

Plasma glutathione S-transferase Alpha 1-1 (GSTA1-1) is a sensitive indicator of hepatocellular damage in various hepatic disorders [3] and the HELLP syndrome [4] To assess possible fetal hepatic damage we measured GSTA1-1 concentrations in maternal blood plasma and corresponding neonatal arterial umbilical plasma samples in nine pregnancies complicated by the HELLP syndrome [1] and in eleven uncomplicated normotensive pregnancies. All pregnancies resulted in caesarean section

Plasma GSTA1-1 levels were measured by an enzyme-linked immunosorbent assay, with an anti-human GSTA mouse monoclonal as catching antibody and rabbit anti-human GSTA antiserum as the source of detecting antibodies [4] The assay has no cross-reactivity with other class GSTs and the variations within and between assays were 2 5% and 7 3%, respectively. Groups of individuals were compared using the Wilcoxon-Mann-Whitney-U test.

Maternal plasma GSTA1-1 levels were much higher in patients than in controls (median 37.7 and 1 3 μg/L, respectively, $P < 0.0001$; Fig 1.). By contrast, babies born to mothers with HELLP syndrome showed similar plasma GSTA1-1 concentrations to those born to controls (4.2 and 5 9 μg/L, respectively). These results suggest that in HELLP syndrome, hepatocellular damage occurs only in the mother, and there is no evidence for neonatal hepatic damage. Toxic substances, oxygen radicals or humoral factors may cause the maternal disease and endothelial damage [5]. Since we found no evidence of hepatocellular damage in neonates born from pregnancies complicated by the HELLP syndrome, those substances either do not seem to cross the fetoplacental barrier, or the fetus may not be susceptible to their effects.

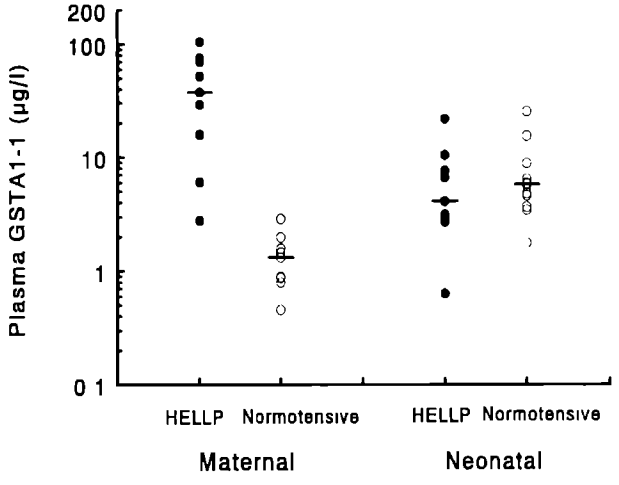

Fig 1 *Maternal and neonatal median plasma GSTA1-1 concentrations in HELLP syndrome (●) and normotensive pregnancy (○)*

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PARAGRAPH 5.5

Plasma glutathione S-transferase Pi 1-1 measurements in the study of haemolysis in hypertensive disorders of pregnancy

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ABSTRACT.

Haemolysis is a major complication of hypertensive disorders of pregnancy, warranting a correct assessment. Lactate dehydrogenase (LDH) and haptoglobin measurements, however, may not accurately reflect its severity. Glutathione S-transferase Pi 1-1 (GSTP1-1) is abundantly present in erythrocytes and its plasma level may theoretically be a marker for haemolysis. Median GSTP1-1 and LDH levels showed to be significantly increased ($P < 0.01$) and haptoglobin significantly decreased ($P < 0.01$) in preeclampsia ($n = 67$) and the HELLP syndrome ($n = 34$) as compared to normotensive pregnancy ($n = 41$). Both GSTP1-1 and LDH levels were significantly higher in normotensive pregnant women as compared to non-pregnant women ($n = 81$; $P < 0.0001$). The percentage of preeclamptic patients or patients with the HELLP syndrome with elevated GSTP1-1 levels was lower than those with elevated LDH or decreased haptoglobin levels. We conclude that plasma GSTP1-1 levels may provide useful information regarding haemolysis in hypertensive disorders of pregnancy in addition to serum LDH activity and plasma haptoglobin levels, and that the degree of haemolysis in hypertensive disorders of pregnancy, especially in the HELLP syndrome, is probably less prominent than generally assumed.

INTRODUCTION.

Pregnancy-induced hypertension, preeclampsia and the syndrome of haemolysis, elevated liver enzymes and low platelets (HELLP) are major causes of maternal and perinatal morbidity and mortality [1] Haemolysis in these multi-system disorders is well recognized as a major complication, especially in the HELLP syndrome [2] and is a predictor for maternal and perinatal outcome in these disorders [3] Because haemolysis is associated with endothelial damage [4], the degree of haemolysis may give an indication of the degree of micro-angiopathy involved. Therefore, a reliable assessment of the level of haemolysis is mandatory. Measurements of serum lactate dehydrogenase (LDH, EC 1.1.1.27) activity may not accurately reflect haemolysis, because, except from erythrocytes, serum LDH may also originate from cardiac and skeletal muscle, kidney, pancreas or the liver. Therefore, a increase in serum LDH activity in hypertensive pregnancies complicated by both hepatocellular damage and haemolysis, such as the HELLP syndrome, could partly be due to hepatocellular damage instead of

haemolysis Until now measurements of serum haptoglobin, the transport protein for haemoglobin, is regarded to be the most reliable indicator of haemolytic disease in non-pregnant patients (diagnostic sensitivity and specificity being 83% and 96%, respectively) [5] and haemolysis in hypertensive disorders of pregnancy [6, 7] However, hepatocellular damage such as in acute hepatitis results in decreased serum haptoglobin levels, probably due to an impaired synthesis [8] Therefore, the coincident hepatocellular damage in hypertensive disorders of pregnancy may give a falsely high impression of haemolysis. Furthermore, haptoglobin levels are undetectably low when a certain level of haemolysis has occurred, which makes the detection of any further increase in haemolysis impossible.

The glutathione S-transferases (GST, EC 2.5.1.18) are a multigene family of enzymes that possess many biologic functions They catalyze the addition of glutathione to a wide variety of compounds such as xenobiotics and serve as intracellular transport proteins for lipophilic compounds [9, 10]. Cytosolic GSTs are dimeric proteins, divided into four classes: Alpha, Mu, Pi and Theta [11] The Pi class GST (GSTP1-1) is found in large quantities in erythrocytes [12, 13], where it is thought to serve as a haemoglobin-binding and transport protein [14] Because of the high GSTP1-1 concentration in erythrocytes, serum GSTP1-1 measurements have been suggested to be a reliable indicator for increased destruction and overproduction of blood cells in certain haematological diseases, such as paroxysmal nocturnal haemoglobinuria [15]

In the present study we investigated plasma GSTP1-1 concentrations in hypertensive disorders of pregnancy, especially the HELLP syndrome as a possible marker of haemolysis as compared to plasma haptoglobin levels and the routinely used serum total LDH activity.

MATERIALS AND METHODS.

Study populations.

Eighty-one healthy non pregnant female blood donors between 20 and 40 years of age, 41 women during uncomplicated normotensive pregnancy, 35 women with pregnancy-induced hypertension, 67 women with preeclampsia and 34 women with the HELLP (Haemolysis Elevated Liver enzymes Low Platelets) syndrome were studied (for population characteristics see Table 1)

Table 1 Population characteristics

	Normotensive pregnancy	*Pregnancy-induced hypertension*	*Preeclampsia*	*HELLP*
Number of patients	41	35	67	34
Maternal age (yr)	32 5 (25 - 41)	31 (21 40)	30* (20 40)	30* (19 39)
Gestational age (wk)	32 5 (27 43)	37* (26 40)	33 (26 42)	30* (18 - 39)
Diastolic blood pressure (K4, mm Hg)	75 (55 -89)	95* (90 130)	100* (90 - 130)	100* (39 125)
Parity	1 (0 3)	0* (0 4)	0* (0 - 3)	0* (0 - 1)

Results are presented as median, range is given in parenthesis
** $P < 0.01$, normotensive pregnancy vs hypertensive pregnancy*

Women entered the study after informed consent was obtained The experimental protocol was approved by the Institutional Review Board of the University Hospital Nijmegen Pregnancy induced hypertension was defined as a diastolic blood pressure ≥ 90 mm Hg on two or more consecutive occasions, each more than 4 hours apart Preeclampsia was defined as pregnancy-induced hypertension and concordant proteinuria (urinary protein/creatinine ratio ≥ 0 30 g/10 mmol) [16] The HELLP syndrome was defined as Haemolysis, defined as increased lactate dehydrogenase activity (> 600 U/L), Elevated Liver Enzymes, defined as increased AST (> 70 U/L) and ALT (> 70 U/L) activities and Low Platelets, defined as a platelet count < 100 x $10^3/mm^3$ [1] Blood pressure was taken in sitting position with a sphygmomanometer Diastole was recorded at phase IV Korotkoff sound

Analytical procedures.

Antecubital venous blood samples were taken in the sitting position after 5 minutes of rest Blood for measurement of plasma GSTP1-1 and haptoglobin levels was drawn in 4 mL EDTA tubes (Becton and Dickinson, Grenoble, France, no 606601) For the measurement of LDH activity blood was collected in 4 mL Corvac vacutainer glass tubes (Monoject, Sherwood Medical, Ballymoney, Northern Ireland, no 37) Serum LDH activity was measured on a Hitachi 747 analyzer (Hitachi, Osaka, Japan), plasma

haptoglobin was determined by rate nephelometry using the CobasFara analyzer (Hoffmann-Laroche, Basel, Switzerland). Both measurements were performed at the local laboratory for clinical chemistry. EDTA blood from 10 healthy staff members was haemolysed by a single freeze-thaw procedure for assessment of haemoglobin and GSTP1-1 concentrations.

Plasma GSTP1-1 concentrations were measured using a recently developed enzyme linked immunosorbent assay [17]. Microtiter plates were coated overnight with purified anti-GSTP1-1 monoclonal antibody. One hundred μl of standard (0 4-100 μg/L GSTP1-1), or 1:1 or 1.100 diluted plasma samples were added to the wells and incubated overnight The next day the plates were washed and incubated with rabbit anti-GSTPi antiserum, washed again, and incubated with horseradish peroxidase labeled swine anti-rabbit antiserum. After a final wash, plates were stained with *o*-phenylenediamine/H_2O_2. Standards and samples were measured in duplicate. A four parameter weighted logistic regression model was used to calculate standard curves and unknowns. The assay has a detection limit of 0.4 μg/L GSTP1-1 and the intra- and inter-assay coefficients of variation are 5.8 % and 10.9 %, respectively.

Statistical analysis.

The statistical significance of differences between median levels of GSTP1-1, haptoglobin concentrations and LDH activity between hypertensive pregnant patients and normotensive pregnant controls and between these normotensive pregnant controls and healthy female blood donors between 20 and 40 years of age was evaluated using the Mann-Whitney U test A *P*-value < 0.05 was considered as statistically significant. In all pregnant patients the correlations between plasma GSTP1-1 and serum LDH, and GSTP1-1 and haptoglobin levels were evaluated using the Spearman's rank coefficient of correlation

Plasma samples were categorized according to their GSTP1-1, LDH and haptoglobin levels being above (elevated) the upper normal reference level (UNRL, being two times the standard deviation above the mean) for GSTP1-1 and LDH and below (decreased) the lower normal reference level (LNRL, being two times the standard deviation below the mean) for haptoglobin. An upper normal reference level of 21.8 μg/L for GSTP1-1 was calculated from plasma samples of 230 healthy blood donors [17] An upper normal reference level of 330 U/L for LDH and a lower normal reference level of 0.40 g/L for haptoglobin was used as specified by the local department of clinical chemistry. All statistical analyses were performed using the Astute' module for Microsoft Excel'

Table 2. Plasma GSTP1-1 and haptoglobin concentration and serum LDH activity

	Blood donors 20 - 40 years	*Normotensive pregnancy*	*Pregnancy-induced hypertension*	*Preeclampsia*	*HELLP*
LDH (U/L)	147 (34 - 305)	215* (129 - 368)	229 (149 - 421)	289# (162 - 1166)	825# (602 - 5980)
Haptoglobin (g/L)	0.92 (0.02 - 2.2)	0 94 (0.14 - 1 8)	0.92 (0.02 - 1.8)	0.5# (0 02 - 1.8)	< 0.02# (< 0.02 -1.2)
GSTP1-1 (μg/L)	7.9 (3 9 - 57 4)	12 5* (6.5 - 50.4)	14.2 (7 8 - 44.8)	15.7# (6.4 - 90.3)	34.5# (6.7 - 281.7)

Results are presented as median; range is given in parenthesis.
 # $P < 0.01$, normotensive pregnancy vs hypertensive pregnancy.
** $P < 0.0001$, normotensive pregnancy vs healthy female blood donors 20 - 40 years.*

Table 3. Percentage of women with abnormal LDH, haptoglobin or GSTP1-1 levels

	Blood donors 20 - 40 years	*Normotensive Pregnancy*	*Pregnancy-induced hypertension*	*Preeclampsia*	*HELLP*
LDH	0%	4.9%	14.3%	38.8%	100%
Haptoglobin	6.2%	7.3%	5 7%	41 8%	97%
GSTP1-1	3.7%	2.4%	14.3%	26.9%	73.5%

UNRL are > 330 U/L, < 0.40 g/L, > 21.8 μg/L for LDH, Haptoglobin and GSTP1-1 respectively.

Results

Whole blood from 10 healthy volunteers (median haemoglobin concentration 8.7 mmol/L) contains extensive amounts of GSTP1-1, median GSTP1-1 plasma level being 4.2 g/L. GSTP1-1 levels and LDH activities were significantly higher in normotensive pregnancies ($P < 0.0001$) as compared to healthy blood donors of the same age (Table 2). Median GSTP1-1 level and LDH activity were significantly higher and plasma haptoglobin levels were significantly lower in preeclampsia and the HELLP syndrome ($P < 0.01$) as compared to normotensive pregnancy (Figs. 1 - 3, Table 2). In all women studied, plasma GSTP1-1 concentrations correlated significantly with serum LDH activity ($r = 0.70$, $P < 0.0001$) and plasma haptoglobin levels ($r = -0.43$, $P < 0 0001$).

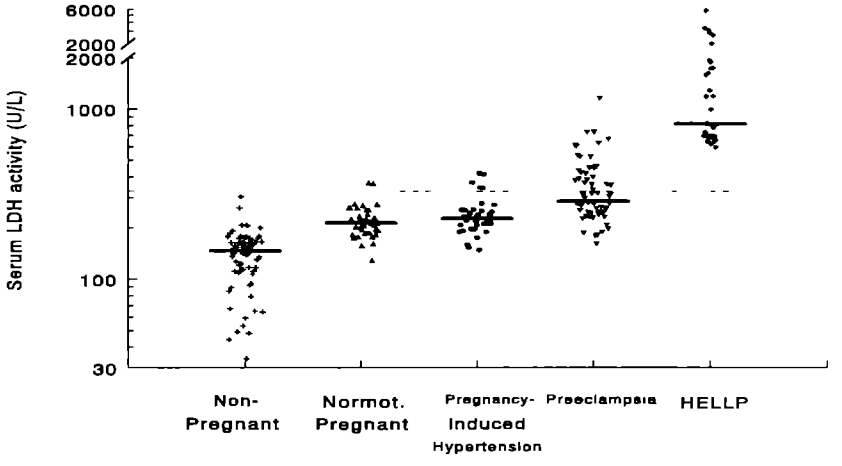

Fig. 1. *Serum LDH activities in healthy female non-pregnant blood donors (+), normotensive pregnancy (▲), pregnancy-induced hypertension (●), preeclampsia (▼) and the HELLP syndrome (◆). The dotted line indicates the UNRL*

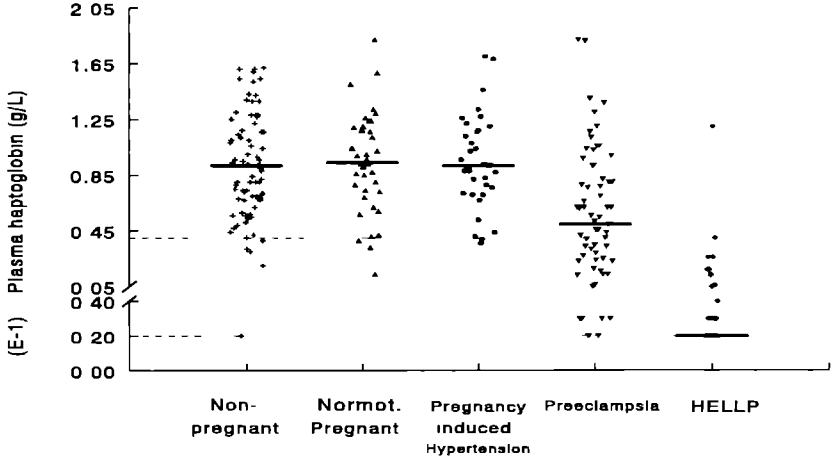

Fig. 2. *Plasma haptoglobin levels in healthy female non-pregnant blood donors (+), normotensive pregnancy (▲), pregnancy-induced hypertension (●), preeclampsia (▼) and the HELLP syndrome (◆). The upper dotted line indicates the LNRL; the lower dotted line indicates the detection limit of the assay.*

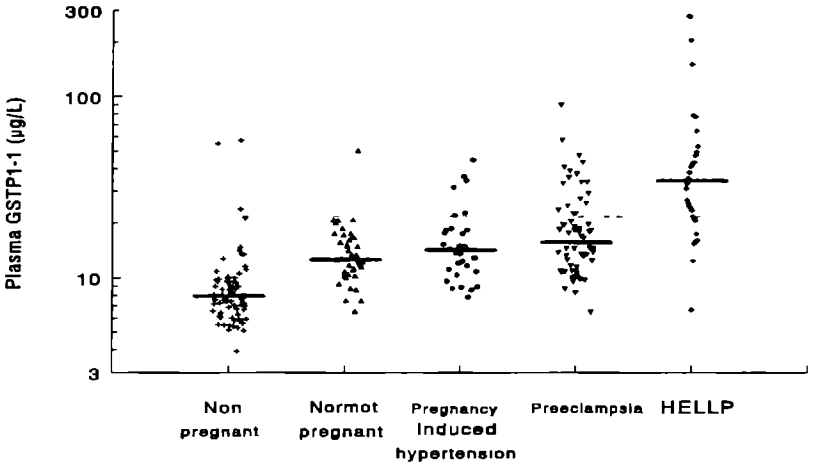

Fig 3 *Plasma GSTP1 1 levels in healthy female non pregnant blood donors (+), normotensive pregnancy (▲), pregnancy-induced hypertension (●), preeclampsia (▼) and the HELLP syndrome (◆) The dotted line indicates the UNRL*

The percentages of women with elevated levels of GSTP1-1 or LDH, or decreased levels of haptoglobin are depicted for each different group in Table 3

Discussion.

Haemolysis in hypertensive disorders of pregnancy, especially the HELLP syndrome is well recognized as a complication, although part of the characteristic elevation of serum LDH activity may as well reflect hepatocellular damage Earlier it has been shown that the observed increase in total serum LDH activity in the HELLP syndrome mainly consists of LDH isotype 5 which predominantly originates from the liver and skeleton muscles, whereas the LDH-1 and LDH-2 isotypes, originating from heart muscle and erythrocytes, showed a moderate absolute increase and even a relative decrease as compared to the LDH-5 isotype [6, 18 - 20] Acute liver injury was reported to lead to a decrease in serum haptoglobin concentrations, probably due to an impaired synthesis of haptoglobin [8] Therefore, the decrease in plasma haptoglobin levels observed in patients with preeclampsia and the HELLP syndrome could be explained by both haemolysis and the acute hepatocellular damage This means that both LDH and

haptoglobin may not be accurate parameters for haemolysis in patients with the HELLP syndrome A peripheral blood smear for assessment of microangiopathic haemolytic disease (i e schistocytes and fragmentocytes) has also been suggested [1], but in analogy with the Haemolytic Uraemic Syndrome, destructed erythrocytes may not be detected when they are already trapped in thrombi and affected arterioles [21]

Because erythrocytes contain abundant amounts of GSTP1-1, measurement of GSTP1-1 plasma levels may more accurately reflect haemolysis, as was earlier suggested in a study on haemolysis in various hematological diseases, such as paroxysmal nocturnal haemoglobinuria and acute leukaemia [15] Normal plasma GSTP1-1 levels, on the other hand, strongly plead against the existence of haemolysis. In the present study we showed that a) median values of GSTP1-1 are significantly elevated in patients with preeclampsia or the HELLP syndrome as compared to normotensive pregnancies, but b) less patients with preeclampsia or the HELLP syndrome showed elevated GSTP1-1 levels as compared to elevated LDH or decreased haptoglobin levels

Because platelets contain GSTP1-1 as well, a rise in plasma GSTP1-1 levels could theoretically not only be due to haemolysis but also due to platelet destruction Because there is actually no evidence in literature that the decrease in platelet count in the HELLP syndrome is due to platelet destruction but only to increased consumption and activation [22], it seems unlikely that platelets significantly contribute to the observed rise in plasma GSTP1-1 levels Although it was suggested that platelet activation may cause an elevation in plasma GSTP1-1 levels [23] this could not be confirmed in methodological studies [17, 24]

In pregnancy serum LDH values are elevated as compared to the non-pregnant situation due to a relative increase in the LDH-4 and LDH-5 isotypes, probably released from the placenta [25] Pregnancy itself showed a significant increase in plasma GSTP1-1 levels (Table 2), which may be due to a release from the placenta Pregnancy-associated rises in serum GSTP1-1 levels were described earlier by Tsuchida *et al* [26]

In conclusion, serum LDH or haptoglobin measurements may overestimate the degree of haemolysis in hypertensive disorders of pregnancy, such as preeclampsia and the HELLP syndrome Plasma glutathione S-transferase Pi 1-1 measurements as a marker for haemolysis during pregnancy deserve further study

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PARAGRAPH 5.6

Decreased glucuronidation of bilirubin as a possible risk factor for the development of the HELLP syndrome

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ABSTRACT

In this study serum liver enzymes and albumin levels were assessed in 54 women 31 (range 3 - 101) months after pregnancies complicated by the HELLP syndrome Serum levels of aminotransferases, lactate dehydrogenase, gamma glutamyl transferase, alkaline phosphatase, albumin and conjugated bilirubin were not elevated Total bilirubin levels, however, were elevated in 20 % of these women, which is significantly different from the prevalence in 151 women with a previous normal pregnancy (χ^2 = 12 23, $P < 0\ 001$), or in the normal female population (χ^2 = 22 34, $P <$ 0 00001) This may suggest that such a dysfunction of the bilirubin conjugating mechanism represents a risk factor for the development of the HELLP syndrome

INTRODUCTION.

The syndrome of haemolysis, elevated liver enzymes and low platelets (HELLP) is a major complication of pregnancy Hepatocellular impairment is one of the hallmarks of this pregnancy-induced disease [1] Usually, signs of liver damage resolve within a few days after termination of pregnancy, although the HELLP syndrome develops postpartum in up to 30 % of cases

In our long term follow up programme of women with pregnancies complicated by the HELLP syndrome we noticed that a significant number of them seems to experience persistence of the upper abdominal pain or fatigue To our knowledge, except for a small study in three women [2], there are no studies concerning the follow-up of hepatocellular integrity at least one month after pregnancies complicated by the HELLP syndrome To investigate whether persistent abdominal pain or fatigue could be related to hepatic dysfunction, serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase, total bilirubin, conjugated bilirubin, gamma glutamyl transferase (γGT), and albumin were assessed in women after pregnancies complicated by the HELLP syndrome

MATERIALS AND METHODS.

Women were selected from the department's archival system in the period of 1 - 1 - 1989 until 1 - 1 - 1996 and subsequently invited for participation in this study The

study protocol was approved by the institutional review board of the University Hospital Nijmegen. The HELLP syndrome was defined as Haemolysis, defined as increased LDH activity (> 600 U/l), Elevated Liver Enzymes, defined as increased AST (> 70 U/l) and ALT (> 70 U/l) activities and Low Platelets, defined as a platelet count < 100 x 10^3/mm^3.

Antecubital venous blood samples were taken in sitting position after 5 minutes of rest in 20 ml 'Corvac' vacutainer glass tubes (Monoject; Sherwood Medical, Ballymoney, Northern Ireland; no 37). All measurements were performed at the local department of clinical chemistry on a Hitachi 747 analyser (Osaka, Japan).

Serum levels of AST, ALT, LDH, γGT, alkaline phosphatase, total bilirubin, conjugated bilirubin and albumin were compared to reference levels used at the local department of clinical chemistry.

Results.

Fifty-four women were recruited. Patient characteristics during the HELLP syndrome were (median, range): gestational age 31 (24 - 40) weeks at delivery, diastolic blood pressure 110 (86 - 140) mm Hg (K4), AST 234 (70 - 2640) U/L, ALT 190 (70 - 910) U/L, LDH 1177 (600 - 7670) U/L and platelet count 52 (10 - 99) x 10^3/mm^3. Eight of these patients developed HELLP syndrome postpartum. At follow up of 31 months

Table Liver function parameters at follow-up of women with a previous HELLP syndrome (n = 54).

	Median (range)	*Reference values*	*Number of women with abnormal values (%)*
AST (U/L)	11 (5 - 38)	< 25	2 (4)
ALT (U/L)	6 (2 - 69)	< 30	1 (2)
LDH (U/L)	209 (120 - 329)	< 330	0 (0)
Alkaline Phosphatase (U/L)	42 (19 - 96)	< 120	0 (0)
Total Bilirubin (μmol/L)	6 (2 - 43)	< 10	11 (20)
Conjugated Bilirubin (μmol/L)	1 (0 - 3)	< 3	2 (4)
γGT (U/L)	10 (6 - 49)	< 35	1 (2)
Albumin (g/L)	45 (39 - 52)	40 - 50	↑ 4 (7), ↓ 1 (2)

postpartum (median, range 3 - 101), serum levels of AST, ALT, LDH, γGT, alkaline phosphatase, conjugated bilirubin and albumin showed to be normal By contrast, eleven out of 54 women (20 %) showed elevated total bilirubin levels (Table).

Discussion.

This study was performed to investigate possible hepatic dysfunction in 54 women who had suffered from the HELLP syndrome. During long time follow-up of these women there were no biochemical signs of impaired hepatocellular integrity, or decreased synthesis capacity of albumin.

Screening for hepatocellular integrity therefore does not seem appropriate. However, we found signs of elevated unconjugated bilirubin levels in a subset of women. Therefore we compared our data with a control group of women from our department, six to eight weeks after an uncomplicated pregnancy, where elevated total bilirubin levels in 7 out of 151 women (5%), and elevated conjugated bilirubin levels in 3 out of 151 women (2%) were found [3]. Data from a large population study were similar for total bilirubin levels which showed to be elevated in 36 out of 745 women (4.8%) [4]. The prevalence of elevated total bilirubin levels in our patient group was significantly higher as compared to the above mentioned control groups (χ^2 = 12.23, $P < 0.0001$ and χ^2 = 22.34, $P < 0.00001$, respectively). These data point to an impairment of the bilirubin conjugating mechanism in one out of five women (20%) with a previous HELLP syndrome. Since no signs of haemolysis were present, according to normal LDH levels, and in the absence of liver disease, unconjugated hyperbilirubinaemia may point to Gilbert's syndrome, which is mainly characterized by an impaired hepatic bilirubin UDP-glucuronyl transferase activity [5]. The prevalence of Gilbert's syndrome in the normal population is 4.8% for women [4]. In addition to the impairment of conjugation of bilirubin, the metabolism of other compounds may also be disturbed in Gilbert's syndrome [6]. The increased prevalence of unconjugated hyperbilirubinaemia, as found in our study, may therefore predispose to the development of conditions such as the HELLP syndrome. This finding warrants further investigation.

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CHAPTER 6

Assessment of glutathione S-transferase Alpha 1-1 levels and aminotransferase activities in umbilical cord blood

PARAGRAPH 6.1

Glutathione S-transferase Alpha 1-1 and aminotransferases in umbilical cord blood

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The assessment of hepatocellular integrity, by means of liver enzyme measurements in umbilical cord blood may contribute to a correct evaluation of the neonatal condition immediately after birth. Aspartate aminotransferase (AST; EC 2.6.1.1) or alanine aminotransferase (ALT; EC 2.6.1.2) levels in blood are the most often assessed clinical markers for hepatocellular integrity. However, elevations of these levels may not specifically indicate hepatic damage for several reasons. There is an apparent lack of AST tissue specificity. Apart from liver tissue AST is found in high concentrations in heart and skeletal muscle, kidney, pancreas and erythrocytes. Haemolysis, for example, significantly contributes to increased serum AST levels. Additionally, AST in liver is present in both mitochondria and cytosol, the majority being present in mitochondria (56% for fetal liver, and 80% for adult liver) [1], whereas hepatic ALT is limited to the cytosol [2]. Acute hepatocellular damage, such as viral hepatitis, results in leakage of mainly cytosolic liver enzymes in the circulation, and the circulating amount of mitochondrial AST is relatively low in these diseases [3]. Serum ALT is considered to be more specific for hepatic damage than AST, because the concentration of ALT in liver is relatively high as compared to other organs. Glutathione S-transferase Alpha 1-1 (GSTA1-1) levels in blood are considered as the most specific and sensitive marker for acute hepatocellular damage in adults [4]. It is rapidly released, in relatively large amounts, into the blood after hepatocellular damage. Because of the short plasma half-life ($\pm$1 hour) its level will follow changes in acute hepatocellular damage more accurately than those of the aminotransferases [4]. In the study of Beckett *et al.* [5], peripheral venous GSTA1-1 levels were suggested to be a more reliable marker for hepatocellular integrity in asphyxiated neonates as compared to ALT. ALT levels did not rise during the first 24 hours after birth complicated by asphyxia, while GSTA1-1 levels were elevated to a maximal level at six hours after birth and declined before ALT levels even rose. Additionally, the magnitude of elevation of GSTA1-1 was higher than that of ALT [5]. Glutathione S-transferases (GST, EC 2.5.1.18) are detoxification enzymes that catalyze the addition of glutathione to a wide variety of xenobiotics. Cytosolic GSTs are dimeric proteins and are divided into four main classes: Alpha, Mu, Pi and Theta [6]. More than 2% of the soluble protein in the liver consists of class Alpha GST. In contrast to the periportal location of aminotransferases, glutathione S-transferase Alpha is primarily located in centrilobular hepatocytes [7]. Two class Alpha subunits have been identified and both homodimers (GSTA1-1 and GSTA2-2) and the heterodimer (GSTA1-2) have been purified from human liver [8].

In the current study three markers for hepatocellular integrity (AST, ALT and GSTA1-1) were studied in arterial and venous umbilical cord plasma, drawn immediately after birth.

Ninety-three arterial and corresponding venous umbilical cord plasma samples were

collected at the Department of Obstetrics and Gynaecology of the 'Nij Smellinghe' Hospital, Drachten, the Netherlands With 49 of them maternal venous plasma samples were obtained as well Arterial and venous umbilical cord blood samples were routinely drawn immediately after birth in preheparinized 2 mL tubes (Kemper Medical BV, Uden, the Netherlands, no 260545) A small volume was used for the assessment of blood gas values on an ABL-330 analyzer (Radiometer Nederland BV, Zoetermeer, the Netherlands) Antecubital maternal venous blood samples were taken in sitting position after 5 minutes of rest in 5 mL heparinized tubes (Becton and Dickinson, Leiden, the Netherlands, no 367684), either less than 4 hours before elective caesarean delivery or less than 15 minutes after vaginal birth Blood was centrifuged within 10 minutes at 1,200 x *g* for 10 minutes and plasma collected Plasma AST and ALT levels were immediately assessed on a Hitachi 704E analyzer (Boehringer Mannheim BV, Almere, the Netherlands) The remainder was stored at -80°C until assessment of GSTA1-1 levels Plasma GSTA1-1 was measured using a recently developed enzyme linked immunosorbent assay [9] The assay has a detection limit of 0.04 μg/L GSTA1 1 and the intra- and inter-assay coefficients of variation are 2 5% and 7 3%, respectively

Groups were compared by the Mann-Whitney U test Correlations were determined by Spearman's rank coefficient of correlation, when appropriate The experimental protocol was approved by the local institutional review board

Both arterial and venous umbilical cord GSTA1-1 and AST levels were significantly higher than corresponding maternal venous levels, whereas ALT was not (Table 1) Arterial umbilical cord plasma AST correlated significantly with corresponding maternal

Table 1 Maternal and neonatal umbilical cord aminotransferase and GSTA1-1 levels (n = 49)

	Maternal venous plasma	*Arterial umbilical cord plasma*	*Venous umbilical cord plasma*
AST (U/L)	10 (1 160)	18 (2 - 45) [a]	15 (1 - 43) [b, c]
ALT (U/L)	3 (1 - 118)	3 (1 21)	3 5 (1 - 18)
GSTA1-1 (μg/L)	1 2 (0 2 - 23 8)	11 2 (1 6 600) [a]	8 3 (1 6 - 550) [d, e]

Results are presented as median (ranges)
[a] *P < 0 0001 arterial umbilical cord vs maternal venous level*
[b] *P = 0 0003 venous umbilical cord vs maternal venous level*
P = 0 002 arterial vs venous umbilical cord level
[d] *P < 0 0001 venous umbilical cord vs maternal venous level*
[e] *P = 0 028 arterial vs venous umbilical cord level*

venous levels (r = 0 32, P = 0 02), whereas ALT and GSTA1-1 did not (r = 0 17, P = 0 24 and r = 0 19, P = 0 20, respectively) Venous umbilical cord plasma AST correlated significantly with corresponding maternal venous levels (r = 0 44, P = 0 0022), whereas ALT and GSTA1-1 levels did not (r = 0 14, P = 0 37 and r = 0 15, P = 0 43, respectively)

Arterial umbilical cord GSTA1-1 correlated significantly with the corresponding AST and ALT levels (r = 0 46, P < 0 0001 and r = 0 41, P < 0 0001, respectively) Arterial umbilical cord AST correlated significantly with corresponding ALT levels (r = 0 58, P < 0 0001)

Arterial umbilical cord plasma GSTA1-1 levels were significantly lower in the caesarean delivery group as compared to the vaginal birth group, whereas no difference was noted for AST or ALT (Table 2) Arterial umbilical cord AST and GSTA1 1 levels correlated significantly with base deficit (r = 0 29, P = 0 005, r = 0 29, P = 0 005, respectively), whereas ALT did not (r = 0 06, P = 0 54) Arterial umbilical cord AST, ALT, and GSTA1-1 levels correlated significantly with birthweight (r = 0 22 P = 0 036, r = 0 47 P = 0 47, and r = 0 26, P = 0 01, respectively)

In this study significantly higher arterial umbilical cord AST and GSTA1 1 but not ALT levels were found as compared to maternal venous plasma levels Earlier, Weiner *et al* [10] found significantly higher AST, but similar ALT levels in umbilical vein specimens obtained for prenatal diagnostic use, as compared to maternal venous levels Maternal AST correlated with umbilical cord AST, whereas maternal ALT and GSTA1-1 did not correlate with their corresponding umbilical cord levels The observed correlation between maternal and umbilical cord AST suggests a relation between both levels Maternal venous AST levels were reported to be higher in pregnancy as compared to the non-pregnant situation, which was suggested to be due to release from placental tissue [10] or uterine muscle [11, 12]

Table 2 Arterial umbilical cord plasma aminotransferase and GSTA1 1 levels in relation to the route of delivery

	Entire group (n = 93)	*Vaginal birth (n = 64)*	*Caesarean delivery (n = 29)*
AST (U/L)	18 (2 - 126)	18 (2 45)	18 (9 126)
ALT (U/L)	4 (1 - 21)	4 (1 21)	5 (1 15)
GSTA1 1 (μg/L)	11 5 (0 6 1360)	13 7 (1 1 1125) *	8 2 (0 6 1360)

Results are presented as median (ranges)
P = 0 002, vaginal birth vs caesarean delivery

Placental AST may be released in the fetal circulation as well thus explaining the correlation between maternal and umbilical cord AST. However, the fact that venous umbilical cord AST is lower than arterial AST pleads against this suggestion There may also be a possibility that fetal AST crosses the feto-placental barrier. Two earlier studies in which fetal umbilical cord blood was obtained during diagnostic umbilical vein puncture in utero, showed that both umbilical cord AST and ALT did not correlate with corresponding maternal levels [10, 13].

Median arterial umbilical cord AST and GSTA1-1, but not ALT levels were significantly higher than corresponding venous levels. The higher arterial AST levels as compared to venous umbilical cord levels are in contrast with the results of Perkins *et al* , who did not find any difference between arterial and venous umbilical cord levels for AST and ALT [14] In previous studies significantly elevated GSTA1-1 [5] and AST [15], but similar ALT levels [5, 15] were observed in peripheral venous blood within 24 hours after uncomplicated delivery, as compared to the period thereafter. The high umbilical cord GSTA1-1 (our study) and the reported high neonatal peripheral venous GSTA1-1 levels [5] were suggested to be due to an increased hepatocellular leakage in fetal and early neonatal life. In addition, lactate dehydrogenase (LDH) was found to be approximately twice as high in neonatal blood as compared to maternal blood [14, 16] mainly due to the rise in the liver-specific LDH5 [16]. This again pleads for high fetal or early neonatal hepatocellular leakage.

The finding of increased arterial umbilical cord GSTA1-1 but not of AST or ALT levels after vaginal birth as compared to those after caesarean delivery, may indicate that the route of delivery has a significant effect on fetal hepatocellular integrity. This could be due to transient periods of hypoxia during uterine contractions which probably more affects the centrilobular, and to a lesser extent the periportal hepatocytes The predominant centrilobular location of GSTA1-1, where the aminotransferases are mainly located periportally, supports this hypothesis. Earlier, Perkins *et al.* noted that the route of delivery did not influence umbilical cord serum AST and ALT levels [14]

Correlations of arterial umbilical cord AST and GSTA1-1 plasma levels with arterial umbilical cord base deficit values were significant, indicating an association between hypoxia or metabolic impairment and hepatocellular integrity. No correlation was found between arterial umbilical cord ALT level and base deficit. Beckett *et al.* did not find an elevation of ALT in neonatal peripheral venous blood in the first few hours following birth complicated by asphyxia [5], but ALT levels were maximally elevated 48 hours after birth. Lehmann *et al* reported no difference in AST levels in umbilical cord blood drawn immediately postpartum of neonates with or without hypoxic events in utero [17] By

contrast, AST levels in peripheral venous samples 24 hours postpartum were significantly higher in neonates with hypoxic events in utero [17]

We established a significant correlation of arterial umbilical cord AST, GSTA1-1 and especially ALT levels with birth weight. These results are in agreement with those of Weiner *et al.* [10], Nava *et al.* [13] and Moniz *et al* [18], who noted a significant increase with advancing gestational age in umbilical cord blood AST and ALT levels. This may be due to increasing hepatic perfusion during gestation as it was shown that GST Alpha activity in fetal hepatocellular tissue does not seem to change from the tenth week of gestation onwards [5, 19, 20].

In conclusion, we studied three potential markers for hepatocellular integrity in umbilical cord blood The correlation between umbilical cord and maternal peripheral venous AST levels makes this parameter unreliable, while ALT does not seem to be sensitive enough to detect early hepatocellular impairment. GSTA1-1 as assessed in neonatal umbilical cord blood, being sensitive and specific, and unrelated to the maternal level, seems to be the most reliable marker for early neonatal hepatocellular integrity and even may detect impaired hepatocellular integrity due to the vaginal birth process Umbilical cord GSTA1-1 may provide a valuable indicator of neonatal condition immediately after birth, the clinical relevance of which needs to be further established

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PARAGRAPH 6.2

Umbilical cord plasma glutathione S-transferase Alpha 1-1 levels as a marker of neonatal hepatocellular integrity

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ABSTRACT.

Objective· To investigate possible delivery-related impaired neonatal hepatocellular integrity by assessment of arterial and venous umbilical cord plasma levels of glutathione S transferase Alpha 1-1 (GSTA1-1)

Methods· GSTA1 1 levels were assessed in arterial and venous umbilical cord and compared to maternal venous plasma samples The influence of maternal, delivery, and neonatal characteristics on arterial umbilical cord GSTA1-1 levels was studied, using linear regression analysis after log-transformation

Results: Median (range) arterial umbilical cord GSTA1-1 plasma levels were higher than venous umbilical cord levels (9 68 [0 64 1125] μg/L and 7 66 [0 78 - 987 5] μg/L, respectively, $P < 0\ 005$) Median (range) arterial and venous umbilical cord GSTA1-1 levels were higher than, and did not correlate with, maternal venous plasma levels (8 79 [1 79 - 183] μg/L and 6 47 [1 58 164 5] μg/L versus 1 47 [0 46 - 10 4] μg/L, $P < 0\ 001$) Neonates born vaginally demonstrated higher median (range) levels than those delivered by caesarean section (13 41 [1 02 1125] μg/L and 5 73 [0 64 172 90] μg/L, respectively, $P < 0\ 001$) Neonates with unfavourable pH (arterial pH under 7 20) demonstrated higher median (range) levels than those with normal pH (arterial pH at least 7 20) (15 15 [0 77 - 1125] μg/L and 8 82 [0 64 - 120 90] μg/L respectively, $P < 0\ 001$) Stepwize multiple linear regression analysis showed that birth weight had the largest influence on arterial umbilical cord GSTA1-1 levels, followed by arterial base deficit, and route of delivery

Conclusion: Arterial umbilical cord GSTA1 1 plasma levels, being unrelated to maternal venous levels, may give a reliable impression of early neonatal hepatocellular integrity and may become an additional indicator of neonatal condition immediately after birth

INTRODUCTION.

The neonatal condition immediately after birth usually is assessed by umbilical cord blood gas analysis and Apgar score estimation These tests do not necessarily reflect impaired integrity of vital organ systems Early assessment of hepatocellular integrity may contribute to a correct evaluation of the neonatal condition immediately after birth

Hepatic function in neonates who have suffered asphyxia at birth usually is assessed by measuring aminotransferase activities in neonatal venous blood However, these activities do not seem to rise during the first hours after birth complicated by asphyxia and, therefore, lack sensitivity in the early postpartum period [1]

The glutathione S-transferases are a multigene family of enzymes that possess many biologic functions They catalyze the addition of glutathione to a wide variety of xenobiotics and serve as intracellular transport proteins for lipophylic compounds [2, 3] Cytosolic glutathione S-transferases (GST) are dimeric proteins, divided into four classes Alpha, Mu, Pi and Theta [4] Two class Alpha subunits have been identified and a heterodimeric (GSTA1-2) and two homodimeric (GSTA1-1 and GSTA2 2) enzymes have been purified from human liver [5] They are released rapidly into the blood after hepatocellular damage [3] in relatively large amounts because the enzyme constitutes as much as 2% of the cytosolic protein in the hepatocyte [6, 7] In contrast to the periportal location of aminotransferases, glutathione S transferase Alpha is primarily located in centrilobular hepatocytes [8] Its half-life in the plasma of adults is approximately 1 hour, and its plasma levels will follow changes in hepatocellular damage more rapidly than aspartate aminotransferase (half life 17 hours) or alanine aminotransferase (half-life 47 hours) [9]

We studied the influence of several neonatal, maternal, and delivery characteristics on neonatal hepatocellular integrity by means of assessment of GSTA1-1 levels in arterial umbilical cord blood plasma immediately after birth Ratios of venous to arterial umbilical cord plasma levels were determined in order to find an indication for a possible concentration-dependent clearance mechanism Umbilical cord plasma levels were compared to birth characteristics (arterial umbilical cord pH, base deficit, bicarbonate [HCO_3], duration of the second stage of labour, and birthweight) and maternal characteristics (age, diastolic blood pressure, and smoking behaviour) The whole group studied was divided in neonates with unfavourable pH (pH under 7 20) or normal neonates (pH at least 7 20) and the vaginal birth group was divided according to the duration of the second stage of labour longer than 60 minutes or 60 minutes or less

Materials and Methods.

Arterial umbilical cord plasma samples were collected from 315 neonates, venous plasma samples from 405 neonates and both arterial and venous plasma samples from 300 neonates at the department of Obstetrics and Gynaecology, University Hospital

Nijmegen, the Netherlands. The experimental protocol was approved by the institutional review board of the University Hospital Nijmegen. Birth, neonatal, and maternal characteristics were collected from maternal patient charts. Except for a subset of 103 women, suffering from various hypertensive disorders of pregnancy known to be associated with impaired hepatocellular integrity, none of the pregnant women had evidence of hepatocellular disease Maternal venous plasma GSTA1-1 levels were measured in 41 women, of whom 16 were suffering from hypertensive disorders of pregnancy with signs of hepatocellular damage and 25 were normotensive without signs of hepatocellular damage.

Arterial and venous umbilical cord blood samples were drawn immediately after birth in preheparinized 1 mL tubes (Smith Industries, Keene, NH). A small volume was used for the assessment of blood gas values. The remainder was stored at 4°C for a maximum of 72 hours Afterward, blood was centrifuged at 11,000 x *g* for 3 minutes and plasma collected. Delayed centrifugation of heparinized blood for a maximum of 72 hours did not influence plasma GSTA1-1 levels, nor were there any differences between heparinized and ethylenediaminetetraacetic acid (EDTA) plasma levels (MFCM-K, unpublished results). Antecubital maternal venous blood samples were collected in 4 mL EDTA tubes (Becton and Dickinson, Grenoble, France), with the subject in the sitting position after 5 minutes of rest, either less than 4 hours before elective caesarean delivery or less than 15 minutes after vaginal birth. Blood was centrifuged within 15 minutes at 3,000 x *g* for 10 minutes and plasma collected. Plasma was stored at -20°C until assessment of GSTA1-1 levels.

Plasma GSTA1-1 levels were measured using a recently developed enzyme linked immunosorbent assay [10] The assay has a detection limit of 0.04 μg/L and the intra-assay and inter-assay coefficients of variation are 2 5% and 7.3%, respectively The ratio of venous to arterial umbilical cord GSTA1-1 levels in groups of increasing arterial levels were tested by one-way analysis of variance with the according Tukey contrast test.

Groups were compared by the Wilcoxon test (pH at least 7.20 versus pH less than 7.20, and duration of the second stage of labour 60 minutes or less versus longer than 60 minutes). The Wilcoxon's test for paired samples was used to test differences between arterial and venous umbilical cord levels and between maternal and umbilical cord levels for statistical significance. Spearman rank correlation coefficients were calculated when appropriate. Bonferoni correction for multiple statistical comparisons was applied to correct for multiple statistical comparisons *P*-values < 0.01 were considered significant

The effects of the maternal and neonatal characteristics on the natural log-transformed arterial GSTA1-1 levels were analysed separately using linear regression techniques. Smoking (no, yes) and route of delivery (vaginal birth, elective caesarean delivery, emergency caesarean delivery) were treated as class variables and all others as continuous variables. The differences were expressed as relative change (with 95% confidence interval [CI]) per unit increase of the characteristic studied (relative change more than 1: increase; relative change less than 1: decrease), calculated from the regression coefficient (with standard deviation) from the model.

Multiple linear regression with stepwize procedures was used to select the maternal and neonatal characteristics that contribute independently to the change of arterial GSTA1-1 plasma levels. Because stepwize procedures do not identify other important characteristics, the *P*-values for entry into the model were considered in order to find close alternatives to the characteristics selected. Differences are expressed as relative changes (95% CI) per unit increase in the characteristic studied, adjusted for the effect of other variables in the model.

Results.

Median (range) maternal characteristics in this study were age 31 (18 - 43) years, parity 1 (0 - 12), and diastolic blood pressure 80 (50 - 149) mm Hg

The median (range) arterial and venous umbilical cord GSTA1-1 plasma level was significantly higher than the median (range) maternal venous plasma level in the 25 normotensive pregnancies studied (8.79 [1.79 - 183] μg/L, and 6 47 [1.58 - 164 5] μg/L versus 1 47 [0.46 - 10.4] μg/L, respectively, $P < 0.001$). No statistically significant correlation was found between either arterial or venous umbilical cord plasma GSTA1-1 levels and maternal venous plasma levels in the group of 41 pairs studied (r = -0.25, P = 0.11; r = -0.03, P = 0.83 respectively), or in the 25 normotensive pregnancies (r = 0.12, P = 0.57; r = 0.23, P = 0.20, respectively).

The median (range) arterial umbilical cord GSTA1-1 plasma level was significantly higher than the median (range) venous umbilical cord plasma level (9 68 [0.64 - 1125] μg/L and 7 66 [0.78 - 987.5] μg/L, respectively; $P < 0.005$; n = 300) and both levels correlated significantly (r = 0 86, $P < 0.001$) The venous-arterial ratio decreased significantly with increasing arterial GSTA1-1 levels (Table 1)

The median (range) arterial umbilical cord plasma GSTA1-1 level was significantly lower after caesarean delivery (n = 98) than after vaginal delivery (n = 217) (5.73 [0 64 - 172.90] μg/L and 13.41 [1.02 - 1125] μg/L, respectively, $P < 0$ 001). The

median (range) arterial umbilical cord level was lower in the elective caesarean delivery group (n = 59) than the emergency caesarean delivery group (n = 39), however this difference did not reach statistical significance (5 02 [0.64 - 25.90] μg/L and 6.58 [0 77 - 172.90] μg/L respectively; P = 0.06).

The median (range) arterial umbilical cord GSTA1-1 level was significantly higher in the group with arterial umbilical cord pH less than 7.20 (n = 76) than in those with pH at least 7.20 (n = 229) (15 15 [0.77 - 1125] μg/L and 8.82 [0.64 - 120.90] μg/L, respectively; $P < 0.001$). The median (range) arterial GSTA1-1 levels were higher in the vaginal birth group with prolonged second stage of labour (n = 37) than in those with normal second stage (n = 160) (19.72 [3.2 - 460.8] μg/L and 12.33 [1 0 - 1125 0] μg/L respectively; P = 0.03), but the difference was not significant. For the use of further analysis we combined the latter two groups into the so-called 'vaginal birth group'.

Table 2 shows the relative change (95% CI) of arterial GSTA1-1 per unit increase of the neonatal or maternal characteristics under study, estimated from the univariate regression models. Significant relative changes were observed for birthweight, arterial pH, base deficit, HCO_3, placental weight, gestational age, and route of delivery Table 3 shows the characteristics that independently influence the arterial umbilical cord GSTA1-1 level. For instance, the estimated relative increase in arterial GSTA1-1 levels is 9 8% per 1 mmol/L increase in base deficit, adjusted for other variables in the model (ie birthweight and route of delivery) Considering the P value for entering in the

Table 1 Venous-arterial ratios of umbilical cord plasma GSTA1-1 levels by increasing arterial levels

Arterial GSTA1-1	*Ratio*	*n*
< 5 μg/L	0 98 (0 50 - 1 82)*	66
5 - 10 μg/L	0.97 (0 51 - 1.21)*	86
10 - 20 μg/L	0 96 (0 18 - 1 34)*	69
20 - 40 μg/L	0 81 (0 08 - 1 41)'	43
≥ 40 μg/L	0 54 (0 03 - 2 75)'	36
Total	0 95 (0 03 - 2 75)	300

Data are presented as median (range)
*, ', ' *same symbol indicates no statistical difference (Tukeys-contrast test, α = 0.05).*

Table 2 Mean relative change of arterial umbilical cord GSTA1 1 levels per unit of the maternal and neonatal characteristics, using linear regression analysis of the log-transformed GSTA1 1 levels

	Relative change (95% CI)	P
Maternal characteristics		
Age (yr)	1 001 (0 97 1 033)	0 943
Smoking		
No	1 00 (reference)	
Yes	0 836 (0 61, 1 156)	0 280
Diastolic blood pressure (mmHg)	0 993 (0 98, 1 003)	0 161
Neonatal characteristics		
Birthweight (kg)	1 832 (1 58, 2 121)	< 0 001
Arterial pH	0 030 (0 00, 0 177)	< 0 001
Arterial base deficit (mmol/L)	1 133 (1 09, 1 177)	< 0 001
Arterial HCO_3 (mmol/L)	0 875 (0 84, 0 912)	< 0 001
Placental weight (g)	1 002 (1 00, 1 003)	< 0 001
Gestational age (d)	1 020 (1 01, 1 025)	< 0 001
Route of delivery		
Vaginal	1 00 (reference)	0 002
Emergency caesarean	0 489 (0 33, 0 720)	0 021
Elective caesarean	0 331 (0 24, 0 459)	0 001

CI = confidence interval, HCO_3 = bicarbonate, GSTA1-1 = glutathione S-transferase Alpha 1 1 In column two, 1 00 indicates no relative change, the relative change of base deficit of 1 133 indicates an increase in arterial umbilical cord GSTA1-1 of 13 3% per unit of increase in base deficit

model we found that alternative variables for birthweight were placental weight and gestational age, for base deficit this was pH and for route of delivery this was HCO_3

Discussion.

We studied the influence of maternal, birth, and neonatal characteristics on umbilical cord GSTA1 1 levels The absence of any correlation between maternal venous and arterial and venous umbilical cord levels as well as the lack of influence of several maternal factors on neonatal levels indicated that maternal factors do not influence

Table 3 Adjusted mean relative change of arterial GSTA1 1 using stepwize selection procedures on all maternal and neonatal characteristics, in a multiple linear regression procedure

	Relative change (95% CI)	*P*-value
Intercept	2 021 (1 23, 3 33)	0 006
Birthweight (kg)*	1 626 (1 41, 1 88)	< 0 001
Arterial base deficit (mmol/L)†	1 098 (1 06, 1 14)	< 0 001
Route of delivery‡		
Vaginal	1 0 (reference)	
Emergency caesarean	0 681 (0 47, 0 98)	0 042
Elective caesarean	0 508 (0 37, 0 69)	< 0 001

CI = confidence interval, HCO_3 = bicarbonate, GSTA1 1 = glutathione S-transferase Alpha 1-1
R^2 = 0 30
In column two, 1 00 indicates no relative change, the relative change of base deficit of 1 098 indicates an increase in arterial umbilical cord GSTA1-1 of 9 8 % per unit of increase in base deficit, adjusted for the other characteristics in the model
* *alternative placental weight, gestational age*
† *alternative pH, (second stage of labour longer than 60 min)*
‡ *alternative HCO_3, (second stage of labour 60 min)*

neonatal levels Therefore, umbilical cord plasma GSTA1-1 levels do seem to reflect neonatal hepatocellular integrity Recent data on pregnancies complicated by the Haemolysis Elevated Liver enzymes Low Platelets syndrome showed that enhanced maternal venous GSTA1-1 levels are not accompanied by elevated arterial umbilical cord levels [11] We found significantly higher umbilical cord GSTA1-1 levels as compared to maternal venous plasma levels These findings are in accordance with those of Beckett *et al.* [1], who found increased levels in peripheral venous blood of newborns after uncomplicated delivery As stated by them, the observed high levels could be due to a higher hepatocellular leakage in fetal and early neonatal life, as a consequence of the premature stage of the hepatocytes [1]

There seems to be some clearance of arterial GSTA1-1 by the placenta because venous umbilical cord levels were significantly lower than arterial levels The clearance mechanism seems to depend on arterial umbilical cord levels, because increasing arterial levels result in lower venous-arterial ratios

The intriguing finding of higher arterial umbilical cord GSTA1 1 levels after vaginal birth as compared to those after caesarean delivery may indicate that the route of delivery has a significant effect on fetal hepatocellular integrity This could be due to either transient periods of hypoxia during uterine contractions or mechanical stress by

uterine contractions and passage through the birth canal.

Arterial umbilical cord pH and base deficit significantly influenced arterial umbilical cord GSTA1-1 plasma levels, possibly indicating decreased fetal hepatocellular integrity due to hypoxia or metabolic impairment. This result is in agreement with the study of Holt *et al* [12] The influence of the pH was slightly lower than that of the base deficit, indicating that a model with the pH is almost similar to a model including base deficit PH reflects acute birth-related hypoxia, whereas base deficit more adequately indicates chronic hypoxia. It seems plausible that chronic hypoxia causes more extensive hepatocellular impairment Hepatocellular impairment related to acute hypoxia in the last period of the second stage of labour may not be reflected in elevated arterial umbilical cord GSTA1-1 levels because some time is required for hypoxia-related breakdown of liverparenchym and subsequent leakage of this enzyme The finding that fetal plasma GSTA1-1 seems to be cleared by the placenta may point to an even higher degree of hepatocellular impairment

The influence of birthweight on arterial umbilical cord GSTA1-1 levels was significant. The increasing level with gestational age may be due to increasing hepatic perfusion during gestation as it was shown that glutathione S-transferase Alpha activity in fetal hepatocellular tissue does not seem to change from the tenth week of gestation onwards [1, 13, 14] A significant increase with advancing gestational age is found for umbilical cord aminotransferase activities as well [15].

In conclusion, we reported detectable GSTA1-1 levels in umbilical cord blood. Levels were associated with birthweight, characteristics indicating intrauterine hypoxic stress, and route of delivery. Assessment of this enzyme may give a reliable impression of early neonatal hepatocellular integrity and may become an additional indicator of neonatal condition immediately after birth

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CHAPTER 7

Effects of hormone replacement therapy on plasma glutathione S-transferase Alpha 1-1 levels in healthy postmenopausal women

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Glutathione S-transferases (GST, EC 2.5.1.18) are detoxification enzymes that catalyze the addition of glutathione to a wide variety of xenobiotics. Cytosolic GSTs are dimeric proteins and are divided into four classes: Alpha, Mu, Pi and Theta [1] More than 2% of the soluble protein in the liver consists of class Alpha GST. These enzymes are released in considerable quantity into the bloodstream during hepatocellular damage Because class Alpha GSTs have a short plasma half-life (±1 hour), its concentration will follow changes in acute hepatocellular damage more accurately than the activities of the aminotransferases [2]. Two class Alpha subunits have been identified and both homodimers (GSTA1-1 and GSTA2-2) and the heterodimer (GSTA1-2) have been purified from human liver [2]

Tiainen and Karhi [3] were the first to report that men had markedly higher plasma GST-Alpha concentrations than women, which was confirmed recently by our group in a much larger study population (median plasma GSTA1-1 concentration in 175 men, 1.92 μg/L and in 175 women, 1.28 μg/L; [4]) However, in women but not in men, a significant increase with age was noted (the median plasma GSTA1-1 concentration in 83 women aged 20-40 years was 1.08 μg/L, in 57 women aged 40-60 years, 1 44 μg/L, and in 33 women above the age of 60 years, 1.84 μg/L; $P<0$ 05 vs women 40-60 and $P<0$ 01 vs women 20-40; [4]) We suggested that a change in hormone concentrations related to menopause may account for the higher concentrations in elderly women [4].

To test the hypothesis that the relatively high plasma GSTA1-1 concentrations in postmenopausal women can be decreased by hormone replacement therapy, these concentrations were measured in 64 healthy postmenopausal women who received hormone replacement therapy during 1 year in a randomized, double-blind, prospective study The study was designed to test reduced frequency of progestogen (norethisterone acetate, NETA) use in a three-monthly hormone replacement therapy (long cycle) vs the standard monthly replacement therapy (Trisequens®). Long-cycle treatment was given during 4 cycles of 84 days, and Trisequens® was given during 12 cycles of 28 days.

Selected subjects were healthy, non-hysterectomized women, aged 45 to 60 years who were amenorrheic for at least 6 months and who had a serum follicle-stimulating hormone concentration higher than 36 U/l and a serum oestradiol below 150 pmol/L All women had serum AST and ALT activities below the upper normal reference limit The 29 women in the long-cycle group received 2 mg of micronized 17β-oestradiol on days 1-78 and 1 mg on days 79-84 and 1 mg of NETA on days 69-78. The 35 women in the Trisequens® group received 2 mg of 17β-oestradiol on days 1-22 and 1 mg on

days 23-28, and 1 mg of NETA on days 13-22.

Plasma samples (K_3EDTA) were collected on three different occasions: (a) at the start of the study (baseline); (b) 1-3 three days before the last NETA administration (17β-oestradiol only; cycle days 66-68 in the long-cycle group and cycle days 10-12 in the Trisequens® group); and (c) on days 8-10 of the last NETA administration (17β-oestradiol + NETA, cycle days 76-78 in the long-cycle group and cycle days 20-22 in the Trisequens® group).

Plasma GSTA1-1 concentrations were measured by sandwich ELISA as reported recently [4] To evaluate the significance of differences between or within two groups, the Mann-Whitney *U*-test and the Wilcoxons matched-pairs signed-rank test were used, respectively This study was approved by the Medical Ethical Review Committee

No significant differences in plasma GSTA1-1 concentrations were detected between the long-cycle and the Trisequens® group before, during, or at the end of the trial period. When compared to those at baseline, plasma GSTA1-1 concentrations were slightly higher ($P=0.20$ for the whole group) at the end of the last 17β-oestradiol-only phase, after almost one year replacement therapy. By contrast, plasma GSTA1-1 concentrations were slightly lower than baseline concentrations at the end of the last 17β-oestradiol + NETA period ($P=0$ 12 for the whole group).

Table Plasma glutathione S-transferase Alpha 1-1 (GSTA1-1) concentrations in postmenopausal women receiving 17β-oestradiol and norethisterone acetate (NETA) in a 28-day (Trisequens®) or a 84-day (long cycle) treatment cycle

	Median plasma GSTA1-1 concentration (μg/l) (range)		
	Trisequens® (n = 35)	Long cycle (n = 29)	All (n = 64)
Baseline	1 84 (0 61-8 27)	2 05 (0.53-5 50)	1 94 (0 53-8 27)
17β-oestradiol only	1 99 (0 46-9 08)	2 31 (0 86-9 35)	2 25 (0 46-9 35)
17β-oestradiol + NETA	1 82[a] (0 39-4 68)	1 58[b] (0 67-4 84)	1 81[c] (0 39-4 84)

Results are presented as median (ranges)
17β oestradiol only vs baseline and 17β-oestradiol + NETA vs baseline were not significantly different
[a] *P<0 03 vs 17β-oestradiol only;* [b] *P<0 003 vs. 17β-oestradiol only;* [c] *P<0 0002 vs 17β-oestradiol only*

In both the Trisequens® group and the long-cycle group, 8-10 days of 17β-oestradiol + NETA resulted in a significantly lower median plasma GSTA1-1 concentration ($P<0.03$ and $P<0.003$, respectively, Table 1). Overall, the last 17β-oestradiol + NETA phase resulted in a 20% decrease of the median plasma GSTA1-1 concentration (in 48 women the plasma GSTA1-1 concentration decreased, in 16 women the plasma GSTA1-1 concentration increased, $P<0.0002$ for the whole group), when compared to 17β-oestradiol only. Sex-related differences in the expression of GST isoenzymes in liver were reported in rats [5] and mice [6] but not in humans. In female mice, ovariectomy resulted in increased liver GST activities that could be normalized by replacement of oestrogen and progesterone [6]. In humans, men had higher plasma GST Alpha concentrations than women [3, 4]. In women, but not in men, an increase with age was noted [4]. This study shows that 17β-oestradiol replacement did not decrease the relatively high plasma GSTA1-1 concentrations detected in elderly women. By contrast, the 8-10 days of replacement with 17β-oestradiol combined with the progestogen NETA resulted in a 20% decrease of the median plasma GSTA1-1 concentration in postmenopausal women, indicating that the relatively high plasma GSTA1-1 concentrations observed in elderly women may be related to the low progestogen concentrations found in these women.

The effect of hormone replacement therapy on plasma GSTA1-1 concentrations in women reported in this study is on the same order of magnitude as the influence of gender and age on plasma GSTA1-1 concentrations. Although the effects of these variables are rather small, they should be kept in mind when plasma GSTA1-1 concentrations of groups of patients are compared to reference intervals obtained from the general population.

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Summary and conclusions

The glutathione / glutathione-related enzyme system in a quantitative sense is one of the most important detoxicating systems in humans. Glutathione and glutathione-related enzymes (glutathione S-transferases and glutathione peroxidases) are involved in the detoxification of endogenous and exogenous toxic compounds, carcinogens, and oxygen radicals. Additionally, the glutathione peroxidases are involved in the metabolism of leukotrienes and prostaglandins Plasma levels of glutathione S-transferase subtypes are markers for disease. Recently, several publications have suggested that deficiencies in this system may be related to the etiology of hypertensive disorders of pregnancy. In 1995, collaborative research on this subject was started at the University Hospital St. Radboud, Nijmegen. In this thesis several studies on the role of the glutathione / glutathione-related enzyme system in hypertensive disorders of pregnancy, and in other gynaecological or obstetrical topics are presented.

In *Chapter 1*, the aim of this thesis is outlined.

In *Chapter 2*, a review of the glutathione / glutathione-related enzyme system, with special reference to gynaecological and obstetrical topics is given

In *Chapter 3*, the results of a study on the presence of glutathione S-transferase Alpha 1-1 (GSTA1-1) and glutathione S-transferase Pi 1-1 (GSTP1-1) in human ovarian preovulatory follicular fluid of patients undergoing an in-vitro fertilization program are presented. Detectable levels of GSTP1-1 and especially GSTA1-1 are demonstrated in follicular fluid, which are not related to concentrations of oestradiol and progesterone.

In *Chapter 4*, the expression of GST Alpha and GST Pi is demonstrated in several tissues from embryos at 8 weeks' gestational age, obtained at surgery for an ectopic twin-pregnancy Both GST Alpha and GST Pi are present in fetal liver tissue, gastrointestinal epithelia, the adrenal gland, and the tela chorioidea of brain tissue. GST Pi, but not GST Alpha is demonstrated in the epithelial tissue of pancreatic ducts and embryonic lung, trachea, nephrons, and urinary collecting ducts, and in the pia mater of brain tissue The presence of these two subtypes of GST, this early in embryonic development, especially in the epithelium of lungs, intestines, and urinary ducts points to the important role of these detoxicating enzymes in protecting the fetus from noxious agents early in development This is important, because several exogenous toxins are known to cross the trophoblast.

In *Chapter 5*, several studies are presented on the role of the glutathione / glutathione-related enzyme system in one of the most important obstetrical disease entities i.e gestational hypertension.

In *Paragraph 5.1*, the glutathione levels, and activities of glutathione S-transferase, selenium-dependent and selenium-independent glutathione peroxidase in placental and decidual tissues of patients with preeclampsia or normotensive pregnancy are investigated. In decidual tissue the glutathione levels are higher than in any human tissue measured previously, both in preeclampsia and in normotensive pregnancy. In preeclampsia, placental and decidual total glutathione levels are significantly higher than those in normotensive pregnancy. In preeclampsia as compared to normotensive pregnancy, the selenium-dependent glutathione peroxidase activity is significantly higher in decidual tissue, whereas selenium-independent glutathione peroxidase activity is significantly higher in placental tissue Total GST activity in preeclampsia is not different from the activity in normotensive pregnancy The observed enhanced detoxicating capacity may point to a compensatory mechanism to protect the mother or the fetus from the increased load of oxygen free radicals or endogenous toxins.

In *Paragraph 5.2*, the maternal whole blood glutathione levels and glutathione / haemoglobin ratios in pregnancies complicated by preeclampsia or the Haemolysis Elevated Liver enzymes Low Platelets syndrome (HELLP) are studied. Maternal whole blood glutathione / haemoglobin ratios are significantly lower in hypertensive pregnancies as compared to those in normotensive uncomplicated pregnancies, suggesting impaired detoxificating or radical scavenging capacity at the time of the disease

In *Paragraph 5.3* results are presented of a study on hepatocellular integrity in hypertensive disorders of pregnancy. This is assessed by measurements of serum alanine aminotransferase (ALT) activity and concentrations of plasma GSTA1-1, a sensitive and specific marker for hepatocellular integrity. GSTA1-1 is shown to be a more sensitive marker for hepatocellular integrity in hypertensive disorders of pregnancy, especially in preeclampsia and the HELLP syndrome, as compared to serum ALT activity Furthermore, when both parameters are evaluated, it is demonstrated that approximately 50% of patients with preeclampsia shows signs of impaired hepatocellular integrity.

In *Paragraph 5.4*, the hepatocellular integrity in neonates born to mothers with the HELLP syndrome is assessed by plasma GSTA1-1 measurements Although the mothers show markedly elevated GSTA1-1 concentrations, their neonates demonstrate physiological concentrations. The HELLP syndrome may be caused by (placenta-derived)

endogenous toxins or oxygen free radicals This study indicates that these substances either do not cross the placenta or the fetus is not susceptible to their effects

In *Paragraph 5 5*, the assessment of haemolysis in hypertensive disorders of pregnancy is evaluated Measurements of serum lactate dehydrogenase (LDH) activity and haptoglobin levels are not specific for haemolysis in hypertensive disorders of pregnancy These multi system disorders are characterized by haemolysis and impaired hepatocellular integrity, the latter resulting in leakage of liver specific LDH into the bloodstream and an impaired production of haptoglobin Hepatocellular impairment may thus lead to a falsely high estimate of the degree of haemolysis Additionally, the assessment of haptoglobin levels as an indirect marker for haemolysis, can not discriminate in the highest ranges of haemolysis due to a rapid depletion of circulating haptoglobin Glutathione S-transferase Pi 1-1 (GSTP1-1) is abundantly present in erythrocytes and is absent in hepatocellular tissue It has been demonstrated a good marker for haemolysis in haematological disease In our study the comparison of LDH, haptoglobin, and GSTP1 1 suggests that the estimation of haemolysis by measurements of GSTP1-1 plasma levels points to a lower degree of haemolysis than the commonly used parameters in hypertensive disorders of pregnancy

In *Paragraph 5 6*, the hepatocellular integrity of non pregnant women who experienced a HELLP syndrome in the past is investigated Both hepatocellular integrity and albumin synthesis capacity is shown to be normal Interestingly, approximately 20% of these former HELLP patients show elevated total serum bilirubin levels, an incidence which is significantly higher than the incidence in the normal population or in a control group of women with uncomplicated obstetrical histories This may point to Gilbert's syndrome, characterized by a dysfunction of the UDP-glucuronyltransferase system which is one of the most important detoxicating systems in humans

In *Chapter 6*, results are presented of two studies on birth related changes in hepatocellular integrity of neonates

In *Paragraph 6 1*, three parameters of neonatal hepatocellular integrity in umbilical cord blood aspartate aminotransferase (AST), ALT, and GSTA1-1 are compared AST is not a specific parameter for hepatocellular integrity, as apart from hepatocellular tissue, it is also found in high concentrations in heart and skeletal muscle kidney, pancreas and erythrocytes Haemolysis, for example, also contributes to increased AST serum levels ALT activity may not be sensitive enough GSTA1 1 is a sensitive and specific marker for hepatocellular integrity as shown in various acute hepatocellular diseases and birth related asphyxia The aminotransferases are periportally distributed in the liver whereas GSTA1-1 is distributed uniformly and therefore important for the

assessment of hypoxia related hepatocellular impairment Although related to base deficit, AST turns out to be of less value because maternal AST correlates with umbilical cord AST activity ALT is relatively insensitive as it does not correlate with markers for hypoxia GSTA1 1 seems to be a reliable marker for birth-related neonatal hepatocellular integrity Interestingly, the route of delivery has a significant influence on GSTA1-1, but not on AST and ALT levels Vaginal birth results in GSTA1-1 levels twice as high as those in caesarean delivery, suggesting a mild hypoxic stress which may affect centrilobular hepatic cells

In *Paragraph 6 2*, umbilical cord GSTA1-1 levels are evaluated in a large group of neonates Results on GSTA1 1 measurements in umbilical cord blood can be confirmed, and multivariate analysis shows significant correlations with markers for hypoxic stress and metabolic impairment There are signs of hepatocellular impairment in groups with unfavourable pH ($< 7\ 20$) at birth as compared to those with normal pH ($\geq 7\ 20$), whereas maternal factors do not influence neonatal umbilical cord GSTA1-1 levels GSTA1-1 may be cleared by the placenta

In *Chapter 7*, results are presented on plasma GSTA1-1 levels in postmenopausal women before, and after hormone replacement therapy Previous studies show that GSTA1-1 significantly increases with age in women, but not in men The progesterone component in HRT is able to reduce plasma GSTA1-1 levels significantly, warranting a cautious interpretation of GSTA1-1 levels in postmenopausal women

Samenvatting en conclusies

Het glutathion / glutathion-gerelateerde enzym systeem is in kwantitatieve zin één van de meest belangrijke ontgiftende systemen van de mens. Glutathion en glutathion-gerelateerde enzymen (glutathion S-transferases en glutathion peroxidases) zijn betrokken bij de ontgifting van endo- en exogene toxinen, kankerverwekkende stoffen en zuurstofradicalen Daarnaast spelen met name de glutathion peroxidases een rol in het metabolisme van leukotrienen en prostaglandines, stoffen van belang voor o.a. de bloedstolling en bloeddruk-regulatie. Bepaalde subtypen van de glutathion S-transferases zijn signaalstoffen voor ziekten Recent hebben verschillende studies de aandacht gericht op mogelijke deficienties van dit systeem bij verschillende vormen van zwangerschapsvergiftiging. Sinds 1995 bestudeert een projectgroep in een samenwerkingsverband tussen de afdeling gynaecologie-verloskunde en het laboratorium van de afdeling maag, darm- en leverziekten van het academisch ziekenhuis Nijmegen de rol van dit systeem bij diverse gynaecologische en verloskundige aandoeningen. In dit proefschrift worden diverse studies gepresenteerd over de rol van dit systeem bij diverse vormen van zwangerschapsvergiftiging, evenals bij andere gynaecologische en verloskundige onderwerpen.

In *hoofdstuk 1* wordt het doel van dit proefschrift uiteengezet

In *hoofdstuk 2* wordt een overzicht van het glutathion / glutathion-gerelateerde enzym systeem gegeven, met name met betrekking tot de rol van dit systeem bij gynaecologische en verloskundige aandoeningen,

In *hoofdstuk 3* worden de resultaten van een studie naar de aanwezigheid van glutathion S-transferase Alpha 1-1 (GSTA1-1) en glutathion S-transferase Pi 1-1 (GSTP1-1) in follikelvocht van patientes die deelnamen aan een in vitro fertilizatie (IVF) programma gepresenteerd GSTA1-1 en met name GSTP1-1 worden in hoge concentraties aangetroffen in follikelvocht, welke niet gerelateerd zijn aan de concentraties van de hormonen progesteron en 17β-oestradiol

In *hoofdstuk 4* worden GST Alpha en GST Pi iso-enzymen aangetoond in zich ontwikkelende organen van 8 weken oude menselijke embryo's, verkregen bij een operatie voor een buitenbaarmoederlijke zwangerschap. Zowel GST Alpha als Pi zijn aanwezig in levercellen, epitheel van weefsel van het spijsverteringskanaal, bijniermerg en in de z g tela chorioidea van de grote hersenen GST Pi, maar niet Alpha werd aangetoond in het epitheel van pancreasklierbuizen en longblaasjes, de luchtpijp, nierlichaampjes, urine-verzamelbuizen en in het zachte hersenvlies van de grote

hersenen De aanwezigheid van deze twee GST-subtypen in deze vroege embryonale ontwikkelingsfase, met name in de bekledende slijmlagen van longen, darmen en urinewegen geven een aanwijzing voor het belang van deze ontgiftende enzymen vroeg in de ontwikkeling Deze resultaten suggereren dat het embryo al vroeg in staat is giftige stoffen onschadelijk te maken.

In *hoofdstuk 5* worden verschillende studies gepresenteerd over de rol van het glutathion / glutathion-gerelateerde enzym systeem bij één van de belangrijkste verloskundige ziektebeelden: zwangerschapshypertensie Zwangerschapshypertensie is een syndromale ziekte, waarbij meerdere orgaansystemen zijn betrokken. De werkelijke oorzaak van de diverse vormen van zwangerschapshypertensie is ondanks veel onderzoek nog onbekend. Mogelijk is er sprake van het ontstaan van een giftige stof of zogenaamde zuurstofradicalen in de placenta (moederkoek), die vrijkomen in de moederlijke bloedsomloop, met als gevolg moederlijke ziekte Immunologische mechanismen kunnen hieraan ten grondslag liggen, danwel een gedeeltelijk ontbrekend, of slecht functionerend ontgiftend systeem.

In *paragraaf 5.1* worden de activiteiten van de glutathion S-transferases en seleniumafhankelijke en onafhankelijke glutathion peroxidases, alsmede concentraties van glutathion in de placenta en de decidua[1] van vrouwen met pre-eclampsie[2] bestudeerd Zowel bij gezonde zwangeren als zwangeren met pre-eclampsie worden in de decidua zeer hoge concentraties glutathion aangetroffen, hoger dan bekend van welk menselijk weefsel dan ook De glutathion concentraties zijn in zowel de placenta als de decidua hoger bij pre-eclamptische zwangerschappen in vergelijking met gezonde zwangerschappen. Bij zwangeren met pre-eclampsie waren zowel de seleniumafhankelijke glutathion peroxidase activiteit in de decidua, als de seleniumonafhankelijke glutathion peroxidase activiteit in de placenta verhoogd, in vergelijking met gezonde zwangerschappen Deze toegenomen 'ontgiftende capaciteit' wijst mogelijk op een compensatiemechanisme ter bescherming van de moeder en het ongeboren kind tegen mogelijke zuurstofradicalen en endogene toxinen, mogelijk geproduceerd door de placenta. Dit compensatiemechanisme is bij deze patienten echter niet toereikend.

In *paragraaf 5.2* worden de glutathion concentraties in het bloed van zwangeren met

[1] slijmvlies dat de baarmoederholte bekleedt tijdens de zwangerschap

[2] zwangerschapsvergiftiging, gekenmerkt door hoge bloeddruk en eiwitverlies in de urine

ernstige pre-eclampsie en/of het HELLP syndroom[3] bestudeerd. De moederlijke glutathion concentraties in deze groep patienten zijn lager dan die van gezonde zwangeren, wat suggereert dat deze zwangeren minder in staat zijn circulerende zuurstofradicalen of giftige stoffen onschadelijk te maken tijdens hun ziekte.

In *paragraaf 5.3* worden de resultaten gepresenteerd van een studie naar leverbeschadiging bij diverse vormen van zwangerschapsvergiftiging, te weten zwangerschapshypertensie, pre-eclampsie en het HELLP syndroom. De leverschade werd geevalueerd middels bepalingen van de alanine aminotransferase (ALAT) en GSTA1-1 concentraties in het bloed. De activiteit van het ALAT in het moederlijke bloed, een enzym dat zich in de lever bevindt en vrijkomt bij leverbeschadiging, wordt beschouwd als de standaard-meting voor leverschade. GSTA1-1 is een ontgiftend enzym dat zich in hoge concentraties in de lever bevindt. Het blijkt dat GSTA1-1 een gevoeligere maat voor leverschade is dan ALAT bij pre-eclampsie en het HELLP syndroom. Als beide metingen worden geevalueerd, blijkt dat er bij méér pre-eclampsie patienten sprake is van leverschade dan gerapporteerd in de literatuur.

In *paragraaf 5.4* wordt m.b.v. GSTA1-1 metingen in navelstrengbloed mogelijke leverschade bij pasgeborenen van moeders met het HELLP syndroom onderzocht. Hoewel de moeders met het HELLP syndroom duidelijk verhoogde GSTA1-1 waarden hadden, waren de GSTA1-1 concentraties van hun kinderen niet verhoogd.

In *paragraaf 5.5* worden drie verschillende metingen van de hemolyse[4] bij zwangerschapshypertensie, pre-eclampsie en het HELLP syndroom vergeleken, te weten. lactaat dehydrogenase (LDH), haptoglobine en GSTP1-1. LDH bevindt zich zowel in rode bloedcellen als in levercellen, dus als LDH als maat voor hemolyse gebruikt wordt bij ziektebeelden die zich tevens kenmerken door levercelverval (zoals b.v. zwangerschapsvergiftiging), kan de mate van hemolyse te hoog ingeschat worden. Ook haptoglobine, een indirecte maat voor hemolyse, kan een vals hoge indruk geven van bloedcelafbraak, omdat bij leverbeschadiging de aanmaak van dit molekuul gestoord is. Tevens kan er geen onderscheid gemaakt worden in de mate van hemolyse als het haptoglobine onmeetbaar laag is. GSTP1-1 bevindt zich in hoge concentraties in rode bloedcellen en komt vrij bij hemolyse. Het bevindt zich niet tot nauwelijks in leverweefsel. Uit deze studie blijkt dat als GSTP1-1 gemeten wordt, er minder sprake van hemolyse lijkt te zijn dan geschat zou worden op grond van LDH en haptoglobine.

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[3] vorm van zwangerschapsvergiftiging, gekenmerkt door afbraak van rode bloedcellen, leverbeschadiging en een afname van het aantal bloedplaatjes in de bloedbaan

[4] afbraak van rode bloedcellen

metingen.

In *paragraaf 5 6* wordt de leverfunctie onderzocht van een groep vrouwen die in het verleden een HELLP syndroom hebben doorgemaakt Het blijkt dat de leverfunctie en de synthesecapaciteit van albumine zich volledig hersteld hebben. Bij 20% van deze voormalige HELLP patienten werden te hoge totaal bilirubine concentraties gevonden, wat een hogere incidentie is dan die in de normale bevolking of in een groep vrouwen, met een ongecompliceerde verloskundige voorgeschiedenis Dit is een aanwijzing dat een deel van deze vrouwen lijdt aan het syndroom van Gilbert, wat zich kenmerkt door een stoornis in het z.g UDP-glucuronyltransferase systeem. Mogelijk kan deze stoornis de ontwikkeling van het HELLP syndroom in de hand werken

In *hoofdstuk 6* worden de resultaten gepresenteerd van twee studies naar aan de geboorte gerelateerde leverbeschadiging bij pasgeborenen.

In *paragraaf 6 1* worden de resultaten gepresenteerd van de vergelijking tussen drie verschillende parameters voor levercelbeschadiging in navelstrengbloed, t.w aspartaat aminotransferase (ASAT), ALAT en GSTA1-1. De ASAT bepaling lijkt minder geschikt, omdat er een relatie bestaat tussen moederlijke en kinderlijke concentraties. De ALAT bepaling lijkt eveneens van minder belang, omdat geen enkele geboorteparameter invloed heeft op de ALAT concentratie. GSTA1-1 leek de meest geschikte parameter, omdat deze duidelijk correleerde met diverse klinische parameters en niet met de moederlijke concentraties. De GSTA1-1 concentraties blijken hoger te zijn bij vaginale bevallingen in vergelijking met die bij keizersneden, terwijl de ASAT en ALAT activiteiten dit verschil niet vertonen. Dit wijst op een lichte mate van levercelbeschadiging als gevolg van de vaginale geboorte, mogelijk als gevolg van een hypoxische beschadiging van het centrale deel van de leverlobjes.

In *paragraaf 6.2* werden de GSTA1-1 concentraties in navelstrengbloed geevalueerd in een grote groep pasgeborenen. De resultaten van paragraaf 6.2 werden bevestigd en een multivariate analyse liet een significant verband zien met andere parameters voor de conditie van het kind, zoals de zuurgraad (pH) en het base-overschot. Er zijn aanwijzingen voor levercelverval bij pasgeborenen met een ongunstige pH ($< 7{,}20$) bij de geboorte, in vergelijking met een groep pasgeborenen met een gunstige pH ($\geq 7{,}20$). Tevens bleek dat moederlijke factoren de navelstrengbloed GSTA1-1 concentraties niet beinvloeden. GSTA1-1 lijkt door de placenta uit het navelstengbloed verwijderd te worden.

Tot slot worden in *hoofstuk 7* de resultaten besproken van GSTA1-1 metingen in bloed van vrouwen in de postmenopauze vóór en na behandeling met hormonale substitutie-therapie. Uit eerdere studies van is gebleken dat de bloedconcentratie van GSTA1-1 bij vrouwen stijgt met toename van de leeftijd, terwijl dit bij mannen niet het

geval was. Dit zou mogelijk een gevolg kunnen zijn van het wegvallen van de beschermende invloed van vrouwelijke geslachtshormonen. Aanvulling van deze hormonen bij vrouwen in de overgang bracht inderdaad een daling teweeg van de bloedconcentraties van deze hormonen, met name na toediening van het hormoon progesteron.

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List of publications

Full papers

1 **Knapen M** Van Schaijk F Mulder T, Peters W, Steegers E Marker for liver damage in neonates born to mothers with HELLP syndrome Lancet 1997,439 519-20

2 Nieuwenhuijzen GAP, **Knapen MFCM**, Oyen WJG, Hendriks Th, Corstens FHM, Goris RJA Organ damage is preceded by changes in vascular permeability in an experimental model of the multiple organ dysfunction syndrome Shock 1997,7 98 104

3 Nieuwenhuijzen GAP, **Knapen MFCM**, Hendriks Th, Van Rooyen N, Goris RJA Elimination of various subpopulations of macrophages and the development of the multiple organ dysfunction syndrome in mice Arch Surg 1997,132 533-9

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Curriculum Vitae auctoris

Maarten Knapen werd geboren op 22 maart 1970 te Helmond. In 1988 haalde hij het eindexamen VWO aan het Carolus Borromeus College te Helmond Vanaf 1989 studeerde hij geneeskunde aan de Katholieke Universiteit Nijmegen Tijdens zijn studie werd in 1993 en 1994 op de afdeling Algemene Heelkunde onder begeleiding van dr G A P Nieuwenhuijzen en dr M J J.M Jansen experimenteel onderzoek verricht naar de pathofysiologie van het Multiple Organ Dysfunction Syndrome, en werd onder begeleiding van dr J A.M Reijnen onderzoek verricht naar het Zuggurtungs-osteosynthese mechanisme bij patellafracturen Tevens werd een onderzoeksstage verricht op de afdeling Chirurgische Pathofysiologie van het Malmo University Hospital, Zweden, waar onder begeleiding van prof A Borgstrom, MD PhD onderzoek verricht werd naar circulerende trypsine Alpha1 antitrypsine complexen bij gezonde vrijwilligers In 1993 werd een stage gelopen bij de ambulancedienst van de GGD Helmond en in 1993 en 1994 was hij werkzaam als student assistent op de afdeling anatomie en embryologie van de medische faculteit In 1995 werd een aanvullend co-schap gelopen in de anaesthesiologie en in 1996 werkte hij met zeer veel genoegen in het Mafeteng Government Hospital, Lesotho, waar een onuitwisbare indruk werd opgedaan van de geneeskunde in een ontwikkelingsland De wetenschappelijk stage werd in 1996 verricht op de afdelingen gynaecologie / verloskunde en gastroenterologie van het Academisch Ziekenhuis Nijmegen onder begeleiding van dr E A P Steegers, dr T P J Mulder en dr W.H M Peters Voor dit werk werd hij in 1997 beloond met de landelijke Hippocrates studieprijs In december 1996 werd het arts-examen behaald (cum laude)

Vanaf november 1996 tot februari 1998 was hij als arts-onderzoeker werkzaam binnen de projecten 'biochemische en haematologische referentiewaarden in de zwangerschap en het kraambed' en 'Onderzoek naar de rol van het glutathion / glutathion gerelateerde enzym systeem in de etiologie van pre-eclampsie en het HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets) syndroom bij zwangeren' Binnen het laatstgenoemde project werden de studies verricht die ten grondslag liggen aan dit proefschrift Van mei tot en met december 1998 zal hij werkzaam zijn als AGNIO op de afdeling gynaecologie / verloskunde van het Canisius-Wilhelmina Ziekenhuis te Nijmegen Op 1 januari 1999 zal hij zijn opleiding tot gynaecoloog aanvangen in het Academisch Ziekenhuis Nijmegen

Curriculum Vitae auctoris

The author of this thesis was born on March 22, 1970 in Helmond, The Netherlands He attended medical school at the Catholic University of Nijmegen, the Netherlands, and graduated cum laude in december 1996 During his medical study he has served as a research assistant at the Departments of General Surgery and Obstetrics and Gynaecology at the University Hospital of Nijmegen, and at the Department of Surgical Pathofysiology, Malmo University Hospital, Sweden In 1997 he was honoured with the Dutch *Hippocrates Prize* for research performed by medical students Additionally he has worked as a teacher at the Department of Anatomy and Embryology, Faculty of Medicine, Catholic University of Nijmegen and he has worked as a junior house officer in Mafeteng Government Hospital, Lesotho From November 1996 till February 1998 he has been a clinical research fellow at the Department of Obstetrics and Gynaecology of the University Hospital of Nijmegen on the studies presented in this thesis, within the project 'Study on the role of the glutathione / glutathione-related enzyme system in the etiology of preeclampsia and the HELLP (Haemolysis Elevated Liver enzymes Low Platelets) syndrome', and on the project 'Biochemical and haematological reference values in uncomplicated pregnancy and the puerperium' From May 1998 until December 1998 he will work as a junior house officer at the Department of Obstetrics and Gynaecology of the Canisius-Wilhelmina Hospital, Nijmegen after which he will start formal training in Obstetrics and Gynaecology at the University Hospital of Nijmegen, the Netherlands

STELLINGEN

behorende bij het proefschrift

THE GLUTATHIONE / GLUTATHIONE-RELATED ENZYME SYSTEM IN REPRODUCTION

Maarten Knapen, 18 september 1998

1 Stellingen over eigen onderzoek kunnen het lezen van het bijbehorende proefschrift voorkomen

2 Een goede samenwerking tussen basale onderzoekers en artsen in de kliniek leidt tot synergisme

3 Samenwerking tussen verloskundige en gynaecoloog (in opleiding) is geen zaak van 'twee geloven op een kussen' maar is gebaseerd op wederzijds respect en vertrouwen

4 Eigenwijsheid is niet altijd wijsheid

5 De periconceptionele periode is meer bepalend voor de uitkomst van de zwangerschap dan alle maanden daaropvolgend

6 Het uitstellen van de eerste zwangerschap en het gebruik van fertiliteitsbevorderende therapieen verhogen de prevalentie van meerlingen in Nederland enorm De Nederlandse bevolking zou hierover geinformeerd dienen te worden
Steegers-Theunissen RPM et al, J Reprod Med 1998,43 173 9

7 Het zg zoutarme dieet voorkómt en geneest zwangerschapshypertensie niet, het voorschrijven hiervan is derhalve zinloos
- Knuist M et al, Br J Obstet Gynaecol 1998,105 430 4, Van Buul BJA et al, Hypertension in Pregnancy 1997,16 335 346

8 In vivo magnetische resonantie spectroscopie (MRS) is mogelijk een nieuwe non-invasieve onderzoeksmethode ter differentiatie van maligne en benigne ovariumtumoren
- Massuger LFAG et al, Cancer 1998,82 1726 30

9 Hyperhomocysteinemie is een onafhankelijke risicofactor voor cardiovasculaire aandoeningen atherosclerotische plaques in de carotisarterie gaan deels in regressie tijdens suppletie van foliumzuur gecombineerd met pyridoxine en vitamine B_{12}
Graham IM et al , JAMA 1997,277 1775-81, Peterson JC et al , Lancet 1998,351 263 -

10 Chance favours the prepared mind
- Louis Pasteur

11 The words used in clinical medicine have a tremendous influence on the subject they describe or purport to describe They perpetuate illnesses, syndromes and signs whose existence is doubtful, they deny recognition to others whose existence is beyond question and, moreover, they distort text-book descriptions to conform to the chosen word
Asher R Richard Asher Talking Sense London Pitman 1972 p 25 -

12 Een typediploma volgens het tienvingersysteem (blind) is eén van de meest waardevolle diploma's voor onderzoekers
- eigen ervaring -

13 Het verrichten van een promotieonderzoek levert een positieve waarde aan de vorming tot medisch specialist, wat niet geevenaard kan worden door een te langdurig AGNIO - arbeidsverleden

14 Doofheid en slechthorendheid zijn sociaal sterk invaliderende en maatschappelijk nauwelijks onderkende ziektebeelden In Nederland lijden een miljoen mensen aan een gehoorverlies van meer dan 25 decibel, 350 000 daarvan dragen een gehoorapparaat van wie de helft ouder is dan 55 jaar
De Volkskrant, 24 - 1 1998 -

15 Een AIO schap wordt zwaar onderbetaald Co-schappen, ook een postdoctorale opleiding, worden gewoon niet betaald

16 Studeren dient voor iedereen financieel mogelijk te blijven, en er dient niet gediscrimineerd te worden op grond van leeftijd

17 All great truths were once blasphemies
George Bernard Shaw